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THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXIII DECEMBER, 1942 No. 6

A HYALURONIDASE AND A POLYSACCHARIDE FROM TUMOURS.

A. PIRIE.*

From the Laboratory of the Imperial Cancer Research Fund, Mill Hill.

Received for publication September 23, 1942.

THE effect of tumour extracts on the spread of vaccinia virus in the skin has been studied by Duran-Reynals and Stewart (1931), and the effect of such extracts on the spread of indian ink and of diphtheria toxin has been investigated by Boyland and McClean (1935). The first authors found that some human epithelial tumours and the Bashford adeno-carcinoma of mice enhanced the vaccinial lesions, whereas other human tumours, mouse sarcoma 37, and chicken tumour No. 1 had no effect. Boyland and McClean (1935) also found that many tumours contained a spreading factor, but their results conflicted at some points with those of Duran-Reynals and Stewart. For instance, they found that mouse sarcoma 37 was the most active tumour of those they tested.

Chain and Duthie (1939) found that the spreading factor is closely associated with hyaluronidase, an enzyme that hydrolyses hyaluronic acid. The test for intradermal spread is much more sensitive than the test for enzyme activity, but it is less specific. Claude (1935) has shown that azoproteins are spreading factors, and McClean (1941) has found that ascorbic acid will act in the same way. An investigation of the hyaluronidase of some tumours has now been carried out and a weak enzyme has been found in rat, mouse, guinea-pig, and in one filterable fowl tumours. Until the naturally occurring spreading factor is proved to be a hyaluronidase the presence of the enzyme does not necessarily mean that the tissue contains an active spreading factor. In general the two have the same distribution, but differences between the relative spreading and hyaluronidase activity in different preparations reported by Mayer, Chaffee, Hobby and Dawson (1941) make it possible that the two are not identical.

The effect of added testis hyaluronidase on the extraction of virus from the Rous and Fujinami tumours has also been studied as these tumours contain no hyaluronidase themselves. The Rous and Fujinami tumours give very viscous extracts which are rapidly made limpid by the addition of hyaluronidase. A viscous polysaccharide has been isolated from these tumours.

* Leverhulme Research Scholar of the Royal College of Surgeons of England.

EXPERIMENTAL METHODS.

Preparation of tumour extracts.

The tumour tissue was frozen immediately on removal from the animal. It was then ground in a cooled mortar and the mush extracted with one or two volumes of distilled water. The tissue was centrifuged down and the supernatant fluid carefully taken off with a pipette. This supernatant was brought to pH 5·0 with dilute acetic acid and was used in the enzyme tests. In the case of the rat, mouse and guinea-pig tumours more than one tumour was used in the preparation of a single extract. The tumours were young tumours obtained from the routine stock carried on in this laboratory. No tumour suspected of being infected was used.

Estimation of hyaluronidase.

The substrate used in testing the activity of hyaluronidase in these tumour extracts was the polysaccharide isolated from Rous and Fujinami tumour tissues. The analysis of this polysaccharide is given in Table III. This shows that it is probably identical with hyaluronic acid and will be referred to as such. The tumour polysaccharide was readily hydrolysed by testis hyaluronidase.

Tubes were set up containing the tumour extract to be investigated, with and without hyaluronic acid. The final concentration of hyaluronic acid in all tests was 0·125 per cent. A few drops of toluene were added to each tube to prevent bacterial growth. Samples of 1·0 ml. were taken at once and pipetted into the deproteinizing agent. The tubes were then incubated at 37° C. and further samples taken after 24 hours and later. The degree of hydrolysis of hyaluronic acid was estimated from the change in the amount of reducing substances which took place during incubation. Any increase in the reducing substances in the control tube was subtracted from the increase in the tube containing hyaluronic acid. There was no change in pH during incubation.

Estimation of reducing sugars.

The samples were deproteinized with cadmium by the method of Fujita and Iwatake (1931). The reducing substances in the supernatant fluids were estimated with ceric sulphate by the method of Giragossintz, Davidson and Kirk (1936).

Boyland and McClean (1935) used a 25 per cent. water extract of frozen ground tissue in their tests of the spreading factor in tumours. Such extracts are very rich in protein and estimations of the reducing sugar content are liable to error in consequence. During incubation these tumour extracts do produce reducing substances, but some of them produce a greater quantity in the presence of hyaluronic acid. Table I shows that all rat, mouse and guinea-pig tumours tested contain a weak hyaluronidase. Of the fowl tumours only Mill Hill I, a slow-growing fibro-sarcoma transmissible with cell free filtrates, shows any activity, and this is very slight. Young Shope papillomas gave inactive extracts. An attempt to confirm the presence of hyaluronidase

in these tumours using a different method of extraction and thus avoiding the large amount of extracted protein gave a negative result in every case. A very active hyaluronidase can be prepared from rabbit testis by extracting the acetone dried tissue with N/10 acetic acid; a 1 per cent. centrifuged extract, which is water clear, contains an active enzyme. Extracts of fowl, rat, mouse, rabbit and guinea-pig tumours made in this way were uniformly inactive. Water extracts of acetone-dried tissue were also inactive.

TABLE I.—*Hydrolysis of Hyaluronic Acid by Tumour Extracts.*

Tumour.	Time of incubation (hours).	Final concentration of extract (%).	Hydrolysis (%).
Mouse 37	24	12·5	35, 38, 30, 15, 0, 0
,, 2146	72	12·5	42
,, 63	24	25	44
Jensen rat sarcoma	48	25	36
Dael cavy	24	10	27
	72	2·5	40
Shope rabbit papilloma	24	14	*Nil* (3 tests).
Mill Hill I fowl fibroma	24	20	11, 25.
Rous sarcoma	48	25	*Nil* (3 tests).
Fujinami myxosarcoma	48	15	,, (3 ,,).

EFFECT OF HYALURONIDASE ON THE ACTIVITY OF EXTRACTS FROM FILTERABLE TUMOURS.

The filterable fowl tumours, Rous No. 1 and Fujinami myxosarcoma contain no hyaluronidase, but contain a large amount of a viscous polysaccharide that is hydrolysed by testis hyaluronidase. Shemin *et al.* (1939) have shown that the hyaluronidase from pneumococci does not destroy the activity of Rous tumour extracts although it rapidly reduces their viscosity. Shemin kept the tumour tissue at 0° C. for 30 min. It seemed possible that by varying the time and conditions of enzyme action the yield of virus from the tissue could be increased. This idea is supported by the fact that lysozyme, which is also a mucinase, can liberate phage from lysogenic and heat-killed bacteria (Wollmann, 1936; White, 1937; Pirie, 1940). Experiments were carried out to see whether incubation of fowl tumour tissue with testis hyaluronidase for different periods and at different temperatures liberated otherwise unextractable virus.

Methods.

1 gm. of fresh tumour tissue was weighed into each of two small sterile mortars, 5 g. of sterile washed sand was added to each and the tissue was ground to a smooth paste. Then 10 ml. of 0·9 per cent. saline and 10 ml. of 0·2 M phosphate buffer pH 6·0 was added to each. To one mortar 2 ml. of 1 per cent. testis extract was added and to the other 2 ml. of distilled water.

The fluids were poured into centrifuge tubes and incubated at different temperature for different times. At intervals samples were taken, neutralized, cooled to 0° C. and centrifuged at 8000 r.p.m. for half an hour. The supernatant fluids were carefully removed, diluted in 0·9 per cent. saline and injected in doses of 0·25 or 0·5 ml. into the breasts or legs of young fowls.

Results.

Table II shows that under no conditions was a significantly greater titre of virus obtained. The viscosity of the extracts was rapidly decreased, but the extraction of virus was not affected. It is interesting that incubation with the enzyme has no deleterious effect on the virus. After one hour's incubation at 37° C. the titre is as great as it was at the beginning of the experiment.

TABLE II.—*Effect of Hyaluronidase on Titre of Tumour Extracts.*

	Dilution of extract.	Incubation of extract.	Tumour sizes. Control extract.	Tumour sizes. Enzyme-treated extract.
Rous	10^{-2}	30′ × 37° C.	++++	++++
	,,	,, ,,	++++	++++
	10^{-3}	,, ,,	+	++++
	,,	,, ,,	−	+
	10^{-4}	,, ,,	−	+
	,,	,, ,,	−	+
Fujinami	10^{-2}	,, ,,	+	+++++
	,,	,, ,,	−	++
	10^{-3}	,, ,,	−	−
	,,	,, ,,	−	−
	10^{-4}	,, ,,	−	−
Rous	10^{-2}	10′ × 18° C.	++++	++++
	,,	,, ,,	++++	+++
	10^{-3}	,, ,,	++++	++++
	,,	,, ,,	++++	+
	10^{-4}	,, ,,	++++	++++
	,,	,, ,,	−	−
Rous	10^{-3}	*Nil*	+++	++++
	,,	,,	+++	++++
	,,	,,	++	+++
	,,	15′ × 37° C.	..	++++
	,,	,, ,,	..	+++
	,,	,, ,,	..	++
	,,	60′ × 37° C.	..	++++
	,,	,, ,,	..	+++
	,,	,, ,,	..	+++

Size of tumour is indicated approximately by the number of "+" signs, the maximum being "++++." Absence of tumour is indicated by "−."

ISOLATION AND PROPERTIES OF THE VISCOUS POLYSACCHARIDE FROM TUMOURS.

Meyer, Chaffee, Hobby and Dawson (1941) showed that hyaluronidase is not a completely specific enzyme. The specificity varies with the source, the testis enzyme hydrolysing chondroitin sulphuric acid and a mucoitin sulphuric acid from cornea as well as hyaluronic acid itself. The viscous polysaccharide of the Rous and Fujinami was isolated mainly as a source of substrate in the study of the hyaluronidase content of tumours, but as several of the preparations contained significant amounts of copper an account of the preparation and of some of the properties of this polysaccharide will be given. Kabat (1939) isolated a polysaccharide similar to and probably identical with hyaluronic acid, from the fluid surrounding a fowl tumour caused by a virus which sometimes caused a sarcoma and sometimes an erythroleucosis. Meyer and Chaffee (1940) isolated hyaluronic acid from the pleural fluid associated with a tumour involving the pleura and peritoneum of a human being.

Both Rous and Fujinami tumours give very viscous extracts. The polysaccharide was isolated from the acetone-dried tissue. Several preparations have been made and the analyses show that the polysaccharide is similar to hyaluronic acid. It contains no sulphur, gives a strong naphthoresorcin reaction for uronic acids, and is rapidly hydrolized by testis hyaluronidase. About 50 per cent. of the available carbohydrate is liberated as reducing sugars by the enzyme.

TABLE III.—*Analysis of Polysaccharide from Fowl Tumour Tissue.*

Preparation number.	Hexosamine (%).	Total reducing sugar (%).	Nitrogen (%).	Acetyl (%).	Sulphur (%).
1	39·4	60·0	3·7	12·5	*Nil*
2	31·3	45·2	3·0	14·8	,,
3	38·8	67·3	4·5	13·7	0·1
Theory	43·1	..	3·37	10·4	*Nil*

The hexosamine was estimated by the method of Elson and Morgan (1933). The total reducing sugars were estimated by ceric sulphate after acid hydrolysis with N H_2SO_4. The results of this estimation are low, as the reducing substances are calculated as glucose, and neither hexosamine nor uronic acids have as great a reducing power as glucose. The nitrogen and acetyl estimations were made by Dr. Weiler of Oxford.

Preparation of polysaccharide.

Acetone dried tumour tissue was extracted with 10 per cent. NaCl on the shaker for some hours. This removed relatively more protein than polysaccharide, but polysaccharide could be isolated fron the extract by prolonged tryptic digestion followed by alcohol precipitation. The residue was extracted overnight with 1 per cent. NaOH. The extract was filtered and neutralized. B.D.H. trypsin to 0·1 per cent. was added to the supernatant fluid, and this

was incubated at 37° C. in the presence of toluene until a test sample gave no precipitate with dilute acetic acid. The fluid was centrifuged and the polysaccharide precipitated with two volumes of alcohol. The stringy precipitate was redissolved in water and centrifuged at 8000 r.p.m. for one hour. The supernatant fluid was carefully taken off, and the process of alcohol precipitation and centrifugation was repeated until the solution was clear and colourless. It was then dialysed, frozen and dried.

Estimation of copper.

Copper was estimated by a colorimetric method, using diethyldithiocarbamate, which is fully described by Keilin and Mann (1938).

One preparation was obviously pale blue when dried, and it contained 0·5 per cent. copper. Further preparations were made in which the water used for all extractions and dialysis was glass distilled and all glassware was washed with glass distilled water before use. These preparations also contained varying and smaller amounts of copper. After precipitation with 12 vols. of glacial acetic acid the copper was removed, but after precipitation with 6 vols. a preparation with 0·1 per cent. copper was obtained. The trypsin used contained no copper.

Mann and Keilin (1938–9) have isolated a copper containing protein from blood and liver. A preparation of protein made from Rous tumour tissue by the method used by Mann and Keilin in their isolation of hepatocuprein contained only traces of copper.

TABLE IV.—*Copper Content of Tumour Polysaccharide.*

Preparation number.	Method of preparation.	Copper. (%).
1	10% NaCl extract	0·52
2	1% NaOH extract	0·09
3	,,	0·12
4	,,	0·14
5	10% NaCl extract	0·02
6	1% NaOH extract	0·08

Robertson, Ropes and Bauer (1941) have shown that ascorbic acid and copper or H_2O_2 together reduce the viscosity of several viscous polysaccharides. Robertson used rather high concentrations of ascorbic acid and copper. McClean (1941) has shown that M/3000 ascorbic acid reduces the viscosity of hyaluronic acid prepared from umbilical cord. He did not find much difference between the effect of ascorbic acid alone and of ascorbic acid plus H_2O_2. A sample of crude umbilical cord hyaluronic acid which Dr. McClean kindly sent to me contained only traces of copper. I have tested the different copper-containing polysaccharides from tumours to see whether the copper they contain can catalyse the viscosity reducing action of ascorbic acid. Even those containing only 0·09 per cent. of copper became limpid on the addition of M/1500 ascorbic acid, whereas the polysaccharide whose copper had been removed by acid precipitation remained viscous until a trace of inorganic

copper was added. Fig. 1 shows the reduction in the flow time of hyaluronic acid in the presence of ascorbic acid. This was measured by noting the time taken for a measured volume, 0·5 ml. to run out of a narrow bore pipette. This method was used owing to the small amount of material available. The results were easily reproducible. Fig. 1 also shows the inhibitory effect of diethyldithiocarbamate, and the effect of the addition of a trace of copper to a mixture of a copper-free polysaccharide and ascorbic acid. The oxidation

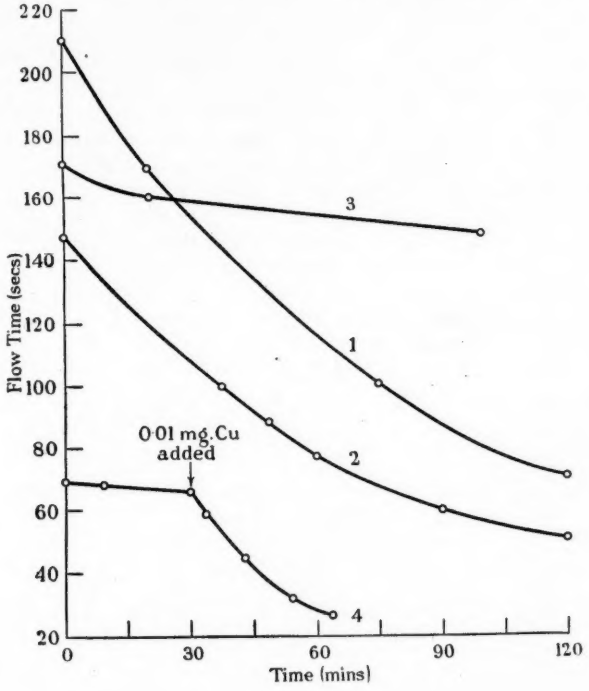

FIG. 1.

Curve 1 : Fraction 6, 0·08 per cent. Cu. + M/3000 ascorbic acid.
,, 2 : ,, 2, 0·12 per cent. Cu. + M/3000 ascorbic acid.
,, 3 : ,, 6, + diethyldithiocarbamate + M/3000 ascorbic acid.
,, 4 : ,, 5, 0·02 per cent. Cu. + M/3000 ascorbic acid. After 30 min. 0·01 mg. Cu. was added.

of ascorbic acid by the copper protein, ascorbic acid oxidase, is also inhibited by diethyldithiocarbamate, Stotz (1940).

The presence of copper in these preparations of polysaccharides may be fortuitous. By the variability of the copper content one can see either that this is so or that an organic copper compound is an impurity in the polysaccharide preparation. Few estimations of the copper content of tumours have been made. Edlbacher and Gerlach (1935) estimated the copper content

of the Jensen rat sarcoma. They found that it was variable, and that necrotic tissue always contained more than the healthy tissue. This is probably due to the fact that this part is drier than the healthy tissue. An average copper content for this tumour was $3 \cdot 87\gamma$/g. The addition of ascorbic acid to a 2 per cent. water extract of frozen dried Rous tumour tissue caused rather a small reduction in the viscosity of the extract. This reduction did not take place without the addition of ascorbic acid, but there may be other oxidizing substances or systems in such an extract which will produce H_2O_2 with ascorbic acid, so that this result is not diagnostic of copper. But it is also possible that the copper and ascorbic acid system has something to do with local changes in the viscosity of tissue hyaluronic acid. It is possible that *in vivo* hyaluronic acid is closely linked with some of the non-dialysable tissue copper, and that this accounts for the high copper content of these tumour polysaccharides.

SUMMARY.

1. Hyaluronidase has been found in some mammalian and one fowl tumour.

2. A viscous polysaccharide has been isolated from two fowl tumours that do not contain hyaluronidase.

3. Preparations of this polysaccharide from tumours contain varying amounts of copper. This copper catalyses a reduction in viscosity of the polysaccharide by ascorbic acid.

I wish to thank Dr. W. E. Gye, F.R.S., for his constant help, and the Royal College of Surgeons for the award of a Leverhulme Scholarship which made this work possible.

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DISTURBANCE OF NUCLEIC ACID METABOLISM PRODUCED BY THERAPEUTIC DOSES OF X AND GAMMA RADIATIONS. PART I: METHODS OF INVESTIGATION.

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Received for publication October 14, 1942.

THESE papers describe a quantitative investigation of the effects of therapeutic doses of X and gamma radiations on the nucleic acid metabolism of normal and malignant cells, using ultraviolet photomicrography as the principal experimental method. The finding of an increase in the absorption of ultraviolet radiation of wavelength 2537 Å by the cytoplasm of cells after X and gamma irradiation has already been recorded (Mitchell, 1940, 1942).

In this investigation a technique of ultraviolet photomicrography is employed in conjunction with objective photometry. This method is based on the work of Caspersson (1936), and was developed quantitatively for the present problem for application to biopsy specimens taken from malignant tumours in accessible sites, e.g. skin, lip, mouth, cervix. The patients are treated with X or gamma radiation under exactly defined routine conditions with special reference to dose and "time factors." From each lesion one biopsy is taken before irradiation and one or more at exact time intervals, e.g. 80 min., 24 hr., after the end of irradiation. The specimens are fixed by a method (Susa-alcohol) selected to minimize changes in ultraviolet absorption. In all cases, *unstained sections* of histologically comparable portions of control and irradiated tissue, which apart from the irradiation have been treated identically, are photographed on the same microscopic field.

For the photomicrography ultraviolet light of the wave-length 2537 Å was selected. The required monochromatic response of the photomicrographic system is attained by employing a mercury-neon discharge tube as light source and a chlorine-bromine filter in conjunction with a suitable unsensitized photographic plate which does not respond to the visible radiation of the wave-lengths greater than approximately 4500 Å, which are transmitted in addition to the 2537 Å line by the layers of chlorine and bromine vapour. The monochromatism has been checked spectro-photographically. The quartz optical system is of conventional type (Köhler and v. Rohr, 1904; Barnard and Welch, 1936) using a dry objective (6 mm. N.A. 0·70). For most of this work a Köhler focusing eye-piece was used, but recently a rapid focusing device employing an optical lever has been developed and found to be very much more efficient. The ultraviolet photomicrographic apparatus is shown diagrammatically in Fig. 1.

* Elmore Research Student.

Objective measurements of the blackening of the photographic plates are made under standard conditions employing a comparison neutral wedge, with standard visible radiation. A microphotometer of non-recording type has been constructed for the measurement of the blackening at selected points on the plates, and in many cases curves have also been obtained by means of a recording microphotometer. From the measurements of the photographic blackening can be deduced the effective concentration at each point in the

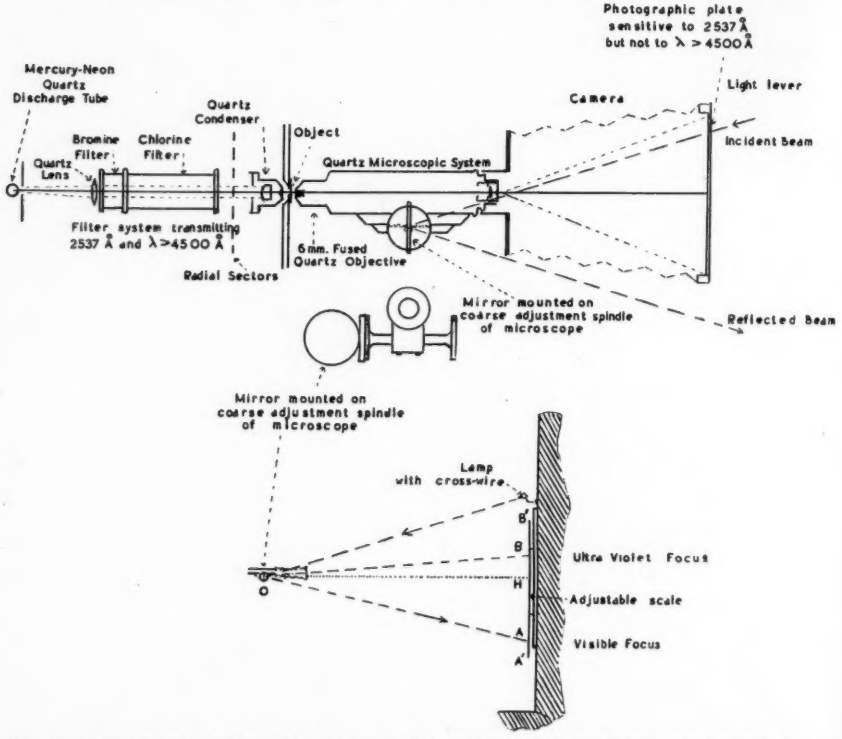

FIG. 1.—Diagram of ultraviolet photomicrographic apparatus showing filters and focusing device.

sections of any chemically identifiable compound, in particular the nucleic acids for which the method has been developed.

An optical interference comparator has been especially developed to measure and correct for the small differences in thickness of the sections of the control and irradiated tissue. Any difference in the light absorption of the two sections due to a difference in the thickness alone is thus estimated and the appropriate, almost invariably small, correction applied to the measured difference in optical density.

The complete ultraviolet absorption spectrum of the substances accumulating in the tissues after irradiation has been measured by two methods, and

in addition it has been possible to measure the approximate spectral distribution of the weak fluorescent radiation due to the accumulating substances.

Histochemical tests have been carried out on the fixed sections, in each case comparing the reactions given by the control and irradiated specimens mounted side by side on the same slide.

SPECIAL EXPERIMENTAL METHODS.

Selection of the Wave-length 2537 Å.

The choice of the wave-length for maximum "optical staining" in ultraviolet photomicrography can be shown* to depend on the ratio of the absorption co-efficients of the nucleic acids and of typical proteins.

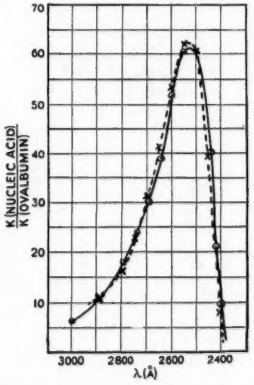

FIG. 2.—Curve showing the variation with wave-length of the ratio of the absorption co-efficients of the nucleic acids and of a typical protein (ovalbumin) in solution.

$$\odot\text{———}\odot \quad \frac{K\ (\text{thymonucleic acid})}{K\ (\text{ovalbumin})}\ \lambda\ \text{max.} = 2525 \pm 20\ \text{Å}.$$

$$\times\text{———}\times \quad \frac{K\ (\text{phytonucleic acid})}{K\ (\text{ovalbumin})}\ \lambda\ \text{max.} = 2530 \pm 20\ \text{Å}.$$

This result determines the selection of the wave-length 2537 Å for the ultraviolet photomicrography.

Both thymo- and phyto-nucleic acids must be considered (Stern and Willheim, 1934; Klein and Beck, 1935). Accurate measurements of the ultraviolet absorption spectra of the nucleic acids in solution have been given by Caspersson (1936). Very many papers have been published on the ultraviolet absorption spectra of typical proteins, e.g. Holiday (1936), but the values selected are from measurements previously made (Mitchell, 1938) for ovalbumin in solution at pH 7·2.

The values of the ratio of the extinction coefficients of 0·1 per cent. solutions of phyto- and thymo-nucleic acids and of ovalbumin at different wave-lengths are given in Fig. 2. The curves show sharply defined maximum values at

* By calculation of the change in photographic blackening due to the interposition in the incident beam of an absorbing layer, which obeys Beer's law to a first approximation as demonstrated *a posteriori*.

the wave-length 2525 ± 20 Å for thymo-nucleic acid and 2530 ± 20 Å for phyto-nucleic acid ; the corresponding values of the ratio of the extinction co-efficients are 62 and 63. With proteins other than ovalbumin the wave-length of this maximum is not significantly altered, although of course the absolute value of the ratio is different, e.g. for thymo-nucleic acid and serum albumin (cf. Caspersson, 1936, fig. 12) the maximum is at approximately 2540 Å and the ratio is approximately 195.

Probably the best value for the wave-length of the maximum is 2530 ± 25 Å, which fortunately is coincident for all practical purposes with the wave-length 2537 Å of the intense and easily obtained mercury resonance line. It must be noted that the wave-length of the maximum of the ratio is considerably less than that of the absorption maximum of the nucleic acids. No evidence has been found for the presence in the cytoplasm of proteins of histone type (Caspersson, 1940), so that these considerations are valid for the quantitative study of cytoplasmic changes.

Filter Method for Producing the Monochromatic Photomicrographic System.

The chlorine-bromine filter for isolating the 2537 Å mercury line was described by Peskov (1917, 1919) ; it has been used frequently in photochemical and other experimental procedures (Forbes, 1928 ; Svedberg and Pedersen, 1940), but it does not appear to have been used previously for ultraviolet photomicrography (cf. Fig. 1). The chlorine filter (Oldenburg, 1924) alone is not adequate. It has been found convenient to mount the two filters as a simple unit, utilizing three optically worked quartz plates as end plates, the rest of the apparatus being of glass. The chlorine filter is a layer, 9 cm. in thickness, of dry chlorine at atmospheric pressure enclosed by stop-cocks (greased with vaseline). The bromine filter is a layer, 2 cm. in thickness, of saturated bromine vapour at atmospheric pressure and is sealed off. The light passing through the filter is rendered parallel at the wave-length 2537 Å by means of the quartz lens.

The combined filter transmits the intense 2537 Å mercury line while effectively eliminating the adjacent lines at 2483 and 2654 Å, but it again transmits in the visible at wave-lengths greater than approximately 4500 Å. Thus it is necessary to use an unsensitized photographic plate which possesses high sensitivity at 2537 Å but does not respond, or has only very low sensitivity, in the visible region transmitted by the filter. The Ilford Zenith plate was found to be suitable ; although this plate still possesses slight sensitivity at 5000 Å, in this region the sensitivity is decreasing rapidly toward longer wave-lengths.

The monochromatism of the photomicrographic response has been demonstrated experimentally by placing the slit of a Hilger large quartz spectrograph in the focal plane of the microscope and photographing the spectrum of the mercury-neon lamp on a Zenith plate. Fig. 3 shows that the intensity of the 4358 Å line, which is the only line detectable in addition to the 2537 Å, is negligible.

The light source is a low-pressure mercury-neon quartz discharge tube similar to that described by Melville (1938) (cf. Rössler and Schönherr, 1938),

but without water cooling of the electrode chambers. The characteristic feature of this type of lamp is the remarkably high intensity of the resonance radiation at 2537 Å.

The microscopic system is of conventional type with the optical axis horizontal. The objective used is a "dry" fused quartz 6 mm. monochromat (Beck) of N.A. 0·70, which, although corrected for the wave-length 2750 Å, can be used at 2537 Å without noticeable loss of definition. With a quartz eye-piece × 14 (Zeiss) the magnification determined photographically by means of an object micrometer is × 670, with camera length 29·0 cm. This value is approximately the upper limit for useful magnification (cf. Drude, 1922, p. 105; Péterfi, 1933, p. 299).

FIG. 3.—Spectrophotographic test of the monochromatism of the optical system for ultraviolet photomicrography with radiation of the wave-length 2537 Å (see text). 1. Spectrum of lamp without filters. 2. Spectrum with chlorine-bromine filter (9 cm. layer of chlorine vapour and 2 cm. layer of bromine vapour). Slit widely open; 5 sec. exposure.

Rapid Focusing Device for Ultraviolet Photomicrography.

The location of the ultraviolet photomicrographic image by means of Köhler's focusing eye-piece is uncertain and extremely time-consuming. To overcome these difficulties, and to give a rapid and accurately reproducible method of measurement of the distance along the optical axis of the microscope between the position of the focus for the visible and the monochromatic ultraviolet radiation, it has been found practicable to measure the necessary small rotation of the coarse adjustment screw of the microscope by means of an optical lever.

The experimental arrangement is shown diagrammatically in Fig. 1. A plane aluminium mirror is mounted in the axis of rotation of the spindle of the coarse adjustment of the microscope. The beam of light from the lamp is incident on the mirror at a point on the axis of rotation and is then reflected on to the adjustable vertical scale; the image of the cross wire of the lamp is focused on to the scale. The scale is graduated in centimetres and is adjustable in the plane A′B′, so that the position of the image of the cross wire corresponding to the visible focus of the microscope is adjusted to the zero mark A; then the coarse adjustment screw of the microscope is rotated so that the image of the cross wire is brought to B, the position of which was determined in the first place by trial and error, using Köhler's eye-piece, and corresponds to the exact setting for the ultraviolet photomicrographic focus. In all cases the quartz slides and cover slips used have been of approximately the same thickness.

Measurements have been made at 2537 Å using the 6 mm. quartz monochromat, at tube length 176 mm. with the quartz eye-piece × 14. The optical axis of the microscope was adjusted to horizontal; in the plane AOB, OH

was measured parallel to the optical axis. For OH = 263·4 cm., AB was found to be 86·0 cm. ; for values of AB differing by ± 0·5 cm. the ultraviolet photomicrographic image was noticeably less sharp. One complete rotation of the mirror corresponded to axial movement of the objective by 16·2 mm. and the angle AOB was 18° 27″, so that the distance between the visible focus and that for 2537 Å was 0·830 mm. This device has been used only during the latter part of this work, see Fig. 15, Part II, and appears reliable for the objective used at the magnification × 670.

Interference Comparator for Measurement of the Difference in Thickness of the Control and Irradiated Sections.

In order to measure the unavoidable small differences in thickness between the control and irradiated sections an interferometric method has been de-

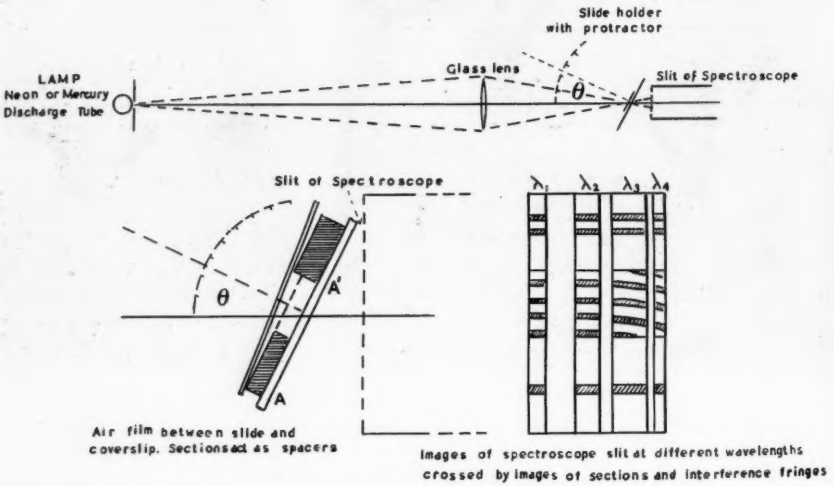

FIG. 4.—Diagram of interference comparator for measurement of difference in thickness of control and irradiated sections.

veloped. The theory of optical interference in plane parallel plates is well known (cf. Rayleigh, 1906 ;. Grebe, 1928 ; Kohlrausch, 1930, pp. 319–327 ; Williams, 1940, pp. 32, 86), and interference comparators are, of course, widely used for the exact measurement of length (Göpel, 1926, p. 1). The present method employs the so-called localized thin plate interference fringes due to difference in thickness, and the experimental arrangements are shown diagrammatically in Fig. 4. For ultraviolet photomicrography the control and irradiated sections are placed in close proximity on a quartz slide. For the present purpose a quartz cover slip is pressed in contact with the upper surfaces of the sections which act as spacers, and optical interference fringes are produced in the air film between the two quartz plates. It can easily be shown

that for interference in either reflected or transmitted light the difference in thickness of the two sections is given by—

$$\Delta t = \frac{\lambda \, \Delta n}{2 \cos \theta}$$

where λ is the wave-length, θ the angle of incidence in the air film, Δn the number of fringes corresponding to AA′. This expression can be regarded as exact for the very small departures from the plane parallel air film which are found under the present experimental condition; e.g. Δt is often of the order 0·1–1μ, while AA′ is of the order 3 mm. In the present experiments it has been found most satisfactory to place the air film immediately in front of the slit of a spectroscope, and focus the radiation from a source giving a sharply defined line spectrum on to the sections. The light source used is either the

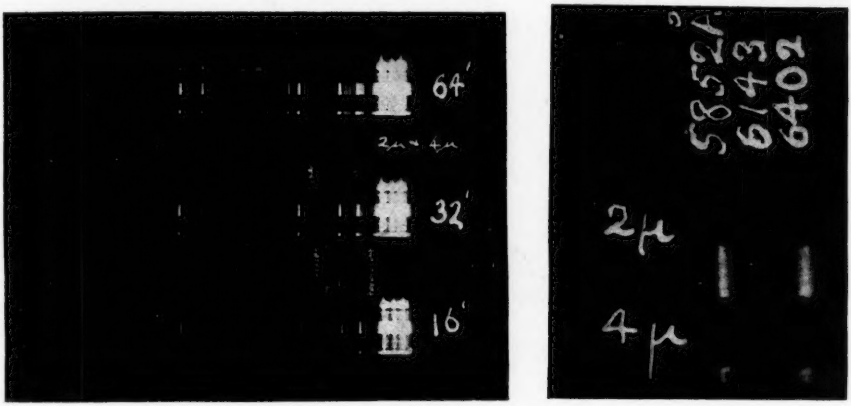

FIG. 5.—Interference comparator. Optical interference fringes produced with sections of nominal thickness 2μ and 4μ as test objects. Measurements on 16 lines of the neon spectrum (3417–6402 Å) gave a value 2·5 ($\pm$ 0·2μ) for the *difference* in thickness of the two sections.

quartz mercury-neon discharge tube used for the ultraviolet microphotography, or a small commercial neon discharge tube which gives the visible neon line spectrum in satisfactory intensity. θ is measured by adjusting the optical axis of the system to horizontal and mounting a small spirit level on the slide carrying the sections, the angle $90° - \theta$ then being measured directly on a graduated scale.

The images of the spectroscope slit at different wave-lengths are crossed by images of the sections and by interference fringes. The fringes are easy to observe, but for accurate measurements must be recorded photographically. The method is used routinely for measurement of the small difference in thickness between the two sections, and to exclude any pairs of specimens showing serious difference in thickness. As test objects sections of nominal thickness, 2 and 4μ, have been examined. Fig. 5 shows the fringes produced with the

neon discharge tube. The mean value $\lambda\Delta n$ for 16 lines of the neon spectrum of wave-lengths 3417–6402 Å (Paschen, 1919) was found to be $4{\cdot}9^3\mu$; $\theta = 11{\cdot}0°$. Thus, the difference in thickness of the two sections $\Delta t = 2{\cdot}5$ ($\pm 0{\cdot}2\mu$). Measurements on another pair of test sections, using the 3663, 4047, 4358 and 5461 Å lines of the mercury spectrum, gave a value $\Delta t = 2{\cdot}4 \pm 0{\cdot}3\mu$. These results appear to provide a satisfactory quantitative test of the method.

Photometry of the Ultraviolet Photomicrographs.

The deflection of the galvanometer used with the microphotometer is measured for standard points on the neutral wedge scale, and for selected marked points on the images of the control and irradiated sections and on the intervening " blank " area. At each point at least four measurements of the galvanometer deflection are made and the mean taken. For histologically comparable areas of the control and irradiated sections at least ten points are usually measured on each and the arithmetic mean of the galvanometer deflections taken for each area.

The galvanometer deflections corresponding to the readings on the neutral wedge scale are plotted graphically against the densities given by the manufacturer's calibration, and from the graph are read off the values of the equivalent visible density corresponding to the mean deflection for the control, blank and irradiated parts of the image. Thus it is possible to evaluate d_{vis} (irradiated) $- d_{vis}$ (control) $= \Delta_{vis}$ and d_{vis} (control) $- d_{vis}$ (blank). From supplementary measurements of the ratio of the gamma values for the visible radiation and ultraviolet radiation of the wave-length 2537 Å, it was found that—

$$\frac{\Delta_{uv}}{\Delta_{vis}} = 1{\cdot}14$$

under the present experimental conditions, so that Δ_{uv} can be deduced directly from the measured galvanometer deflections. The percentage increase in optical density of the control section after irradiation is obtained directly from the ratio—

$$\frac{\Delta_{vis}}{d_{vis}\ (\text{control}) - d_{vis}\ (\text{blank})}$$

and comparison of this value with the measured difference in thickness of the two sections shows immediately whether any significant error is introduced by the difference in the thickness of the specimens. In every case the individual plate measurements are subjected to statistical analysis, the mean galvanometer deflections for the individual points selected for the control and irradiated sides being compared as small samples; the significance of the measured difference between the control and irradiated sections is assessed by using " Student's " t-distribution and calculating P_s (as defined by Yule and Kendall, 1937, p. 439).

The experimental error in the measurement of $\Delta_{uv} = d_{uv}$ (irr.) $- d_{uv}$ (control) is minimized by making the measurements on the two sections at points as close together as possible and at least 1 cm. from the edges of the plate. No fog correction appears to be necessary. The maximal experimental error in

the measurement of Δ_{uv} is estimated at 0·04 (cf. Twyman and Allsopp, 1934, pp. 51, 52 ; Svedberg and Pedersen, 1940, p. 251).

Methods of Measurement of the Ultraviolet Absorption Spectra of the Histological Preparations.

I. *Macroscopic method.*

The difference in *mean* optical density of the control and irradiated sections has been measured using the Hilger Spekker photometer in conjunction with a large quartz spectrograph. A parallel slit of adjustable width was inserted into the two beams of the Spekker photometer. The control and irradiated sections (or ribbons of sections placed side by side), immersed in liquid paraffin and mounted between quartz cover slips, were placed so as to cover the effective apertures, which are approximately 7 mm. long. (Any small gaps in the sections are filled in with indian ink.) It is evident that on account of inhomogeneity, this method gives invariably lower values for d (irradiated) — d (control) than those obtained by the following method.

II. *Spectroscopic image method.*

This approximate method has the advantage that the density difference measurements are made on selected homogeneous areas of the sections of

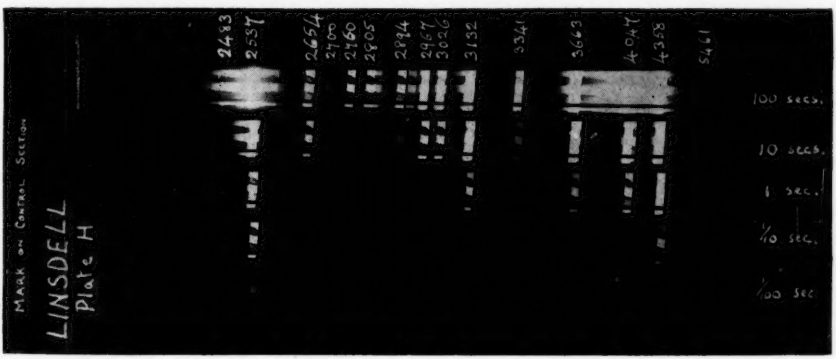

FIG. 6.—Plate illustrating the approximate method of measurement of the difference between the ultraviolet absorption spectra of the control and irradiated sections. (Case 3, X-ray series.)

dimensions $\frac{1}{2}$–1 mm. It depends on photometric measurements of the photographic blackening of the images at a number of wave-lengths for a series of photographs with either different exposure times, or the same exposure times with different intensities produced by means of a radiating sector (see Fig. 6). For a given small density difference to a first approximation under the present experimental conditions—

$$d_2 - d_1 = \frac{1}{\gamma} \log \frac{\theta(2)}{\theta(1)}$$

where $\theta(1)$ and $\theta(2)$ are the corresponding galvanometer deflections. By variation of intensity or exposure time, the value of $\frac{\theta(2)}{\theta(1)}$ passes through a maximum corresponding to the linear part of the characteristic curve. Thus for each wave-length in the line spectrum of the radiation used, despite the wide variations in intensities at different wave-lengths, it is possible to deduce from the photometric measurements of the photographic images in the focal plane of the spectrograph the small difference in density of the two sections. The accuracy of this method is low, but its validity has been checked with known substances.

Radiological Details.

The primary X-radiation used was of effective wave-length approximately 133 X.U. corresponding to H.V.L. = 1·39 mm. copper (Grebe and Nitzge, 1930; Mayneord and Roberts, 1935). The apparatus was a "200 K.V. Maximar" working at 196 K.V.p. and 10·0 m.a. with F.S.D. 40 cm. The additional filter employed was 1 mm. copper + 1 mm. aluminium. The apparatus was calibrated, using a rice phantom and a Victoreen dosimeter and the measurements were kindly checked by Dr. D. E. Lea, using a free air ionization chamber. All measurements given include back scatter and the field margins were defined by lead rubber apertures on the surface.

The gamma ray treatments were carried out in most cases using radium on individually moulded applicators. Case 14 was treated by means of a radon seed implant, the seeds being removed after 192 hr. and the distribution being checked by radiographs. Case 15 received standard Stockholm radium treatment. The radium moulds were similar to those described by Murdoch, Simon and Stahel (1930) and Paterson and MacVicar (1937), but for simplicity were constructed entirely of "black tray compound." The doses were calculated using the data published by Paterson and Parker (1934), or in some cases directly using the formulae and tables given by Sievert (1930, 1932). The filtration was 0·5 mm. Pt. It is assumed that 1 Imc. hr. = 8·4 r and the homogeneity of the field at the skin was checked photographically. The effective wave-length of the primary gamma radiation is approximately 16 X.U. (Bruzau, 1929; Mayneord and Honeyburne, 1938).

SUMMARY.

This paper describes the experimental methods employed in a quantitative investigation of the effects of X and gamma radiations on the nucleic acid metabolism of normal tissues and malignant tumours by means of ultraviolet photomicrography. An essential feature is the photometric comparison on the same photomicrographic field of fixed unstained sections of biopsy specimens of control and irradiated tissue which apart from the irradiation have been treated identically. Additional information is provided by measurements of the ultraviolet absorption and fluorescence spectra and by histochemical tests. A number of new experimental methods have been devised.

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DISTURBANCE OF NUCLEIC ACID METABOLISM PRODUCED BY THERAPEUTIC DOSES OF X AND GAMMA RADIATIONS. PART II: ACCUMULATION OF PENTOSE NUCLEOTIDES IN CYTOPLASM AFTER IRRADIATION.

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Increase in Cytoplasmic Absorption at 2537 Å.

A striking increase in the ultraviolet absorption of the cytoplasm of cells after irradiation has been found in 11 out of 15 pairs of biopsy specimens from gamma-ray treated cases, and in 13 out of 17 pairs of biopsy specimens from

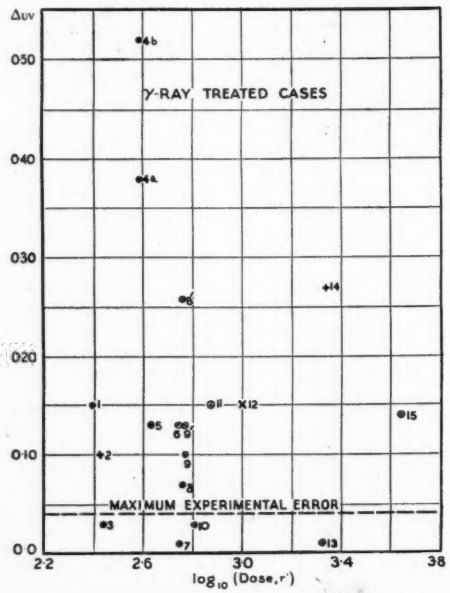

FIG. 1.—Measurements on gamma-ray treated cases. Graph showing dependence of increase in ultraviolet absorption of cytoplasm (Δ_{uv}) upon *dose*. (The numbers refer to the case numbers in the series.)

\+ Biopsy 0 min. after end of irradiation.
○ ,, 80 ,, ,, ,, ,,
× ,, 9 hr. ,, ,, ,,
— — — — Maximum experimental error.

*Elmore Research Student.

cases treated with X radiation. The histology of the tissues examined is given in Table I, and the quantitative experimental results are summarized in Figs. 1, 2, 3, 4, and 5.*

The order of magnitude of the observed increase in the optical density of the cytoplasm for absorption of ultraviolet radiation of wave-length 2537 Å is 0·15 for sections of approximate thickness 2μ. The interpretation of this will be discussed below, but it may be noted here that this increase in density

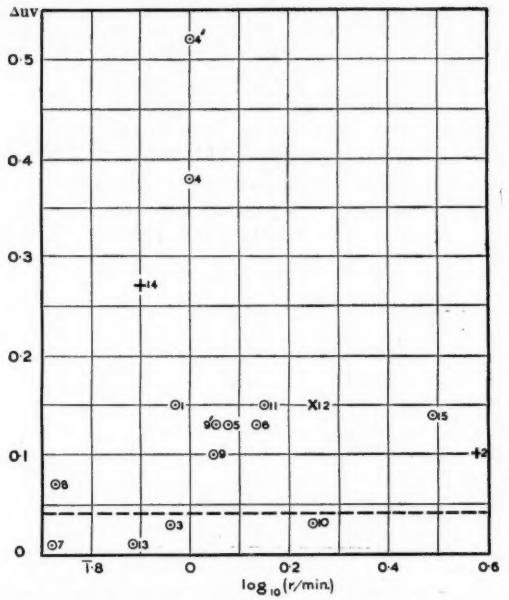

FIG. 2.—Measurements of gamma-ray treated cases. Graph showing dependence of increase in ultraviolet absorption of cytoplasm (Δ_{uv}) upon dose rate. (The numbers refer to the case numbers in the series.)

\+ Biopsy 0 min. after end of irradiation
⊙ ,, 80 ,, ,, ,, ,,
× ,, 9 hr. ,, ,, ,,
— — — — Maximum experimental error.

corresponds to a local concentration in the cytoplasm of the affected cells of 3·5 per cent. of phyto-nucleic acid or the corresponding nucleotides; the absorbing material is not thymonucleic acid as shown by the negative Feulgen and Dische reactions (cf. Dische, 1930; Lison, 1936).

The increase in cytoplasmic absorption after irradiation is shown typically by proliferating and differentiating cells; with fully differentiated cells there may be a small change,† but typically there is no change. As yet no quantitatively significant differences in behaviour have been observed between the

* The author will be pleased to supply these results in tabular form to anyone interested.

† The possibility of diffusion of absorbing substances of low molecular weight introduces uncertainty with regard to the histological localization of small changes.

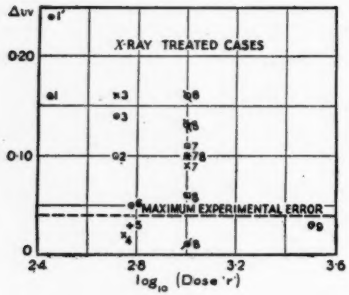

FIG. 3.

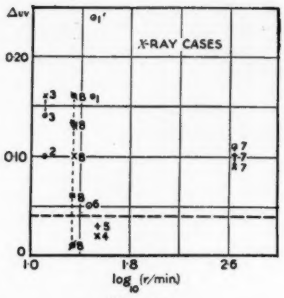

FIG. 4.

FIG. 3.—Measurements of X-ray treated cases. Graph showing dependence of increase in ultraviolet absorption of cytoplasm (Δ_{uv}) upon *dose*. (The numbers refer to the case numbers in the series.)

⊙ Biopsy 80 min. after end of irradiation.
\+ ,, 18 hr. ,, ,, ,,
× ,, 24 ,, ,, ,, ,,
⊙ ,, 7 days ,, ,, ,,
† ,, 14 ,, ,, ,, ,,
⊙ ,, 30 ,, ,, ,, ,,
⊡ ,, 56 ,, ,, ,, ,,
— — — — Maximum experimental error.

FIG. 4.—Measurements on X-ray treated cases. Graph showing dependence of increase in ultraviolet absorption of cytoplasm (Δ_{uv}) upon dose rate. (The numbers refer to the case numbers in the series.)

⊙ Biopsy 80 min. after end of irradiation.
\+ ,, 18 hr. ,, ,, ,,
× ,, 24 ,, ,, ,, ,,
⊙ ,, 7 days ,, ,, ,,
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— — — — Maximum experimental error.

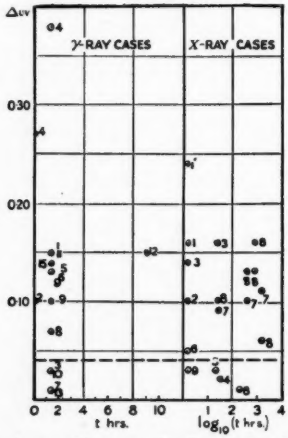

FIG. 5.—Graphs showing dependence of increase in ultraviolet absorption of cytoplasm (Δ_{uv}) upon time of biopsy after end of irradiation. (The numbers refer to the case numbers in the series.)

t hr. : Time of biopsy after end of irradiation.

TABLE I.—*Gamma-ray Treated Cases.*

Case No.	Histology of structures examined.
1	Hyperplastic keratinizing squamous epithelium adjacent to keratinizing squamous-cell carcinoma of lower lip.
2	Normal skin of tadpole.
3	Hyperplastic keratinizing squamous epithelium adjacent to squamous-cell carcinoma of lower lip.
4	Hyperplastic keratinizing squamous epithelium continuous with squamous-cell carcinoma of lower lip.
5	Hyperplastic keratinizing squamous epithelium continuous with squamous-cell carcinoma of ear.
6	Keratinizing squamous-cell carcinoma of lower lip.
7	Hyperplastic keratinizing squamous epithelium continuous with squamous-cell carcinoma of scalp.
8	Hyperplastic keratinizing squamous epithelium adjacent to basal-cell carcinoma of scalp.
9	Hyperplastic keratinizing squamous epithelium adjacent to basal-cell carcinoma of skin of temporal region.
10	Hyperplastic keratinizing squamous epithelium continuous with keratinizing squamous-cell carcinoma of face.
11	Basal-cell carcinoma of skin of face.
12	Anaplastic squamous-cell carcinoma of cervix.
13	Keratinizing squamous-cell carcinoma of lower lip.
14	Hyperplastic keratinizing squamous epithelium adjacent to basal-cell carcinoma of skin of outer canthus.
15	Squamous-cell carcinoma of cervix.

X-ray Treated Cases.

1	Hyperplastic keratinizing squamous epithelium adjacent to squamous-cell carcinoma of skin of temporal region.
2	Squamous-cell carcinoma of fauces.
3	*a*. Normal squamous epithelium of hard palate adjacent to— *b*. Large spindle-cell sarcoma of antrum.
4	Keratinizing squamous-cell carcinoma of lower lip.
5	Hyperplastic keratinizing squamous epithelium adjacent to squamous-cell carcinoma of lower lip.
6	Hyperplastic keratinizing squamous epithelium adjacent to anaplastic carcinoma of skin of thigh.
7	Squamous-cell carcinoma of vulva.
8	Keratinizing squamous carcinoma of vulva.
9	Squamous-cell carcinoma of fauces.

cells of the malignant tumours examined—mostly squamous and basal-cell carcinomata and one large spindle-cell sarcoma—and the homologous cells of the adjacent normal and hyperplastic tissues. Further, a similar increase in ultraviolet absorption has been observed with living cells in culture (chick heart and choroid fibroblasts and skin epithelial cells) irradiated in situ on the microscope stage by means of a radium applicator placed on the objective, giving a dose of 340 r of gamma radiation in one hour.*

Typical changes with normal squamous epithelium are shown in Figs. 6 and 7 (Case 3a). These sections show a striking increase in ultraviolet absorption, $\Delta_{uv} = 0{\cdot}14$, 80 min. after 516 r X-radiation in 40 min. (Fig. 6).

* These experiments have been made in conjunction with Dr. A. W. Calm and Dr. I. Glücksmann, and if possible will be described later.

These photographs show clearly the great increase in cytoplasmic absorption in the growing cells of the stratum basale and the differentiating cells of the stratum spinosum. The microphotographs at magnification × 670 and the microphotometer tracings show that the cytoplasmic absorption increase is very much greater in the incompletely differentiated cells than in those almost fully differentiated. Further, the microphotometer tracing shows that the concentration of absorbing substances accumulating after irradiation is greatest immediately outside the nuclear membrane and decreases toward the periphery of the cytoplasm (cf. Caspersson, 1940, fig. 5). Fig. 7, Case 3a, shows comparable biopsy specimens from the same case before and 24 hr. after 516 r in 40 min. Measurements show that in the areas of more completely differentiated cells the absorption increase persists without significant change, but that there is very considerable recovery in the more undifferentiated cells.

The changes shown by *hyperplastic epithelium* adjacent to squamous-cell carcinomata are shown in Figs. 8–11 (Case 1) and by the hyperplastic epithelium adjacent to basal-cell carcinomata of the skin in Fig. 14 (Case 8). (All these cases were treated with gamma radiation.) In all instances there is an obvious and quantitatively significant increase in the ultraviolet absorption of the cytoplasm.

The changes shown by *squamous-cell carcinomata* are exemplified by Figs. 12 and 13 (Case 8) (X-ray treatments).

Case 12 provided sections from a recurrent anaplastic squamous-cell carcinoma of the cervix before and 9 hr. after 1000 r of gamma radiation given in 9·47 hr. by means of a specially constructed applicator. The measurements made on the comparable areas of tumour tissue show that $\Delta_{uv} = 0{\cdot}15$, and

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DESCRIPTION OF PLATES.

FIGS. 6 and 7.—Case 3a. Normal squamous epithelium of hard palate. × 300.
6. Sections: Control and 80 min. after 516 r in 40 min. ($\Delta_{uv} = 0{\cdot}14$).
7. Sections: Control and 24 hr. after 516 r in 40 min. ($\Delta_{uv} = 0{\cdot}16$).

FIGS. 8–11.—Case 1. Hyperplastic epithelium adjacent to squamous-cell carcinoma of lower lip, treated with double radium mould. × 300. Sections: Control and 80 min. after 248 r in 4·45 hr. The observed increase in cytoplasmic absorption at 2537 Å ($\Delta_{uv} = 0{\cdot}15$) corresponds to that of a 3·5 per cent. solution of phytonucleic acid or its constituent ribo-nucleotides.
8. Unmarked plate.
9. Plate marked for measurement with non-recording microphotometer.
10. Plate marked for measurement with recording microphotometer.
11. Disappearance of cytoplasmic absorption after acid hydrolysis and extraction with absolute alcohol.

FIGS. 12 and 13.—Case 8. Keratinizing squamous carcinoma of vulva. × 300.
12. Sections: Control and 14 days after 1000 r at 22 r/min. ($\Delta_{uv} = 0{\cdot}13$).
13. Sections: Control and 30 days after 1000 r at 22 r/min.

FIG. 14.—Case 8. Hyperplastic epithelium adjacent to basal-cell carcinoma of scalp. × 300. Sections: Control and 80 min. after 575 r in 18·0 hr. ($\Delta_{uv} = 0{\cdot}07$—a significant increase). Measurements on marked plate show no change in the nucleic acid content of the nuclei.

FIG. 15.—Case 3b. Large spindle-cell sarcoma of antrum. × 300. Sections: Control and 80 min. after 516 r in 40 min.

FIGS. 16 and 17.—Photomicrographs showing increase in fluorescence after gamma irradiation. Case 1. Squamous-cell carcinoma of lip. Sections: Control and 80 min. after 248 r in 4·45 hr.
16. Fluorescence with exciting radiation of wave-length 2537 Å.
17. „ „ „ „ 2950–4250 Å.

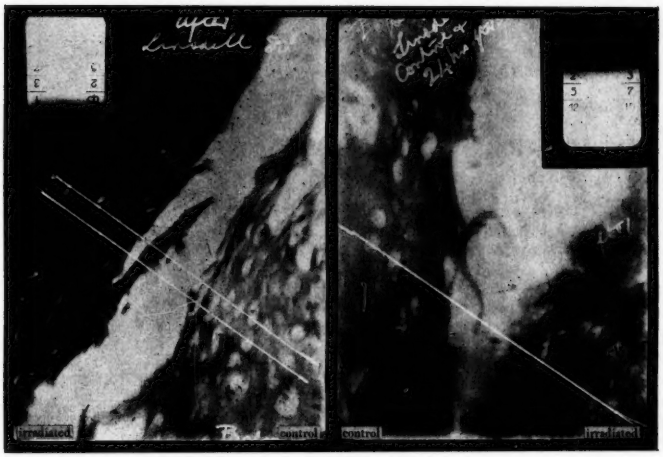

FIG. 6.

FIG. 7.

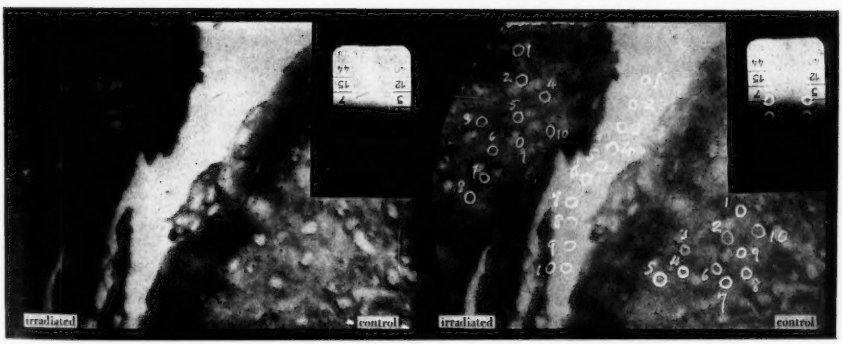

FIG. 8.

FIG. 9.

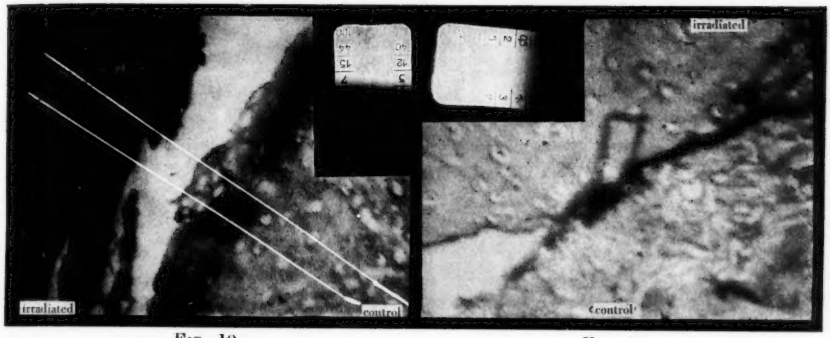

FIG. 10. FIG. 11.

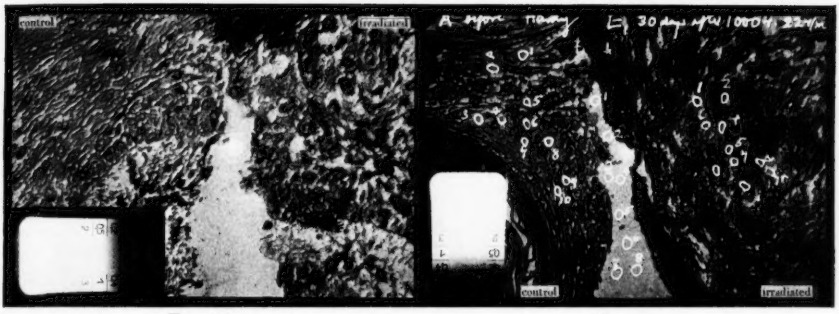

FIG. 12. FIG. 13.

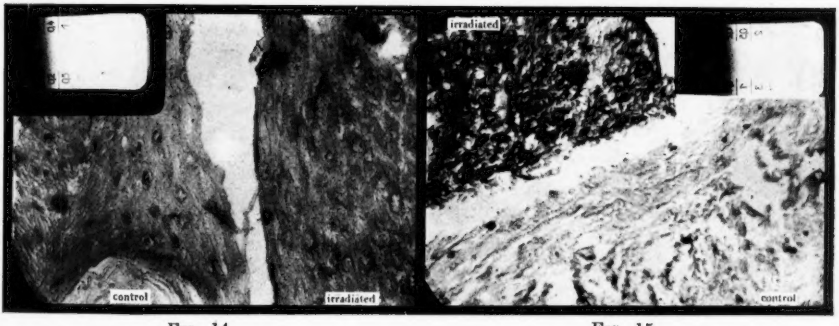

FIG. 14. FIG. 15.

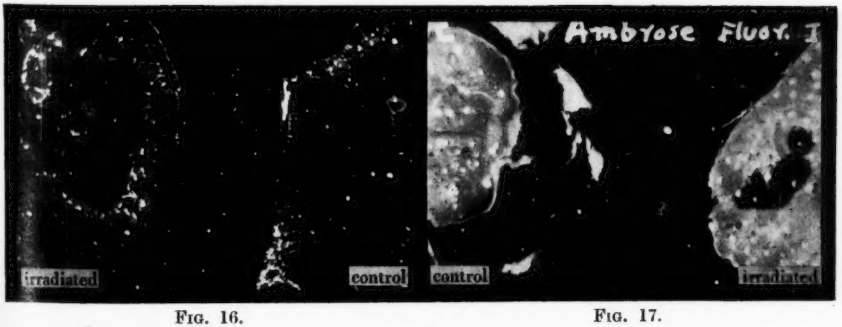

FIG. 16. FIG. 17.

it is evident that recovery has been far from complete during the 9-hr. interval following irradiation. Examples of the changes observed after X-ray treatment of carcinoma of the vulva are shown in Figs. 12, 13 (Case 8), and similar measurements were also made with Case 7. In both these cases the clinical response was unsatisfactory, and it is of interest that in Case 7 the cytoplasmic absorption increase persists without significant change 56 days after 1000 r X-radiation given at 444 r/min. Similarly with Case 8 the absorption increase persists for 30 days, but there is evidence of recovery after 56 days.

Microphotographs of a large spindle-cell *sarcoma* of the antrum are shown in Fig. 15 (Case 3b), and demonstrate a very striking increase of cytoplasmic absorption; $\Delta_{uv} = 0{\cdot}26$ in the tumour cells 80 min. after 516 r given in 40 min.

The dependence of the increase in ultraviolet absorption of the cytoplasm (Δ_{uv}) upon the dose is shown for the gamma-treated cases in Fig. 1 and for the X-ray-treated cases in Fig. 3. The increase in ultraviolet absorption does not increase with the dose of radiation, being approximately constant for single doses ranging from 250–4400 r units of gamma radiation and 280–1000 r units of X-radiation. It is evident that the " saturation " values for Δ_{uv} are already reached at the smallest doses examined. The dose rate appears to be a factor of importance—see Figs. 2 and 4—the increase in cytoplasmic absorption showing approximately constant values for dose rates 1·00–444 r/min., but low values at dose rates less than 0·9 r/min. with gamma radiation. In this discussion the great experimental error renders unnecessary the introduction of the wave-length effect factor (cf. Mitchell, 1940). In Fig. 5, giving the dependence of Δ_{uv} upon the time of the biopsy after the end of irradiation, is shown the great variation in the rate of recovery processes ; in typical radiosensitive cases approximately complete recovery occurs after 24 hours. It is of interest to note that the cytoplasmic absorption increase can persist unchanged even for as long as 56 days, and it is tempting to suggest that this failure of metabolic recovery may perhaps be correlated with clinical radioresistance.

Ultraviolet Absorption Spectrum of Accumulating Substances.

Fig. 18 curve (1) shows the *approximate* spectrum of the increase in optical density of the sections of a large spindle-cell sarcoma of the antrum at 80 min. after 516 r X-radiation (Case 3). The sections were of nominal thickness 6μ, and the measured difference in thickness of the control and irradiated sections was less than 0·3μ. The measurements in Fig. 18 were made by the spectroscopic image method and refer to selected small homogeneous areas of the tumour.

Fig. 19 shows the " difference absorption spectrum " for Cases 1 and 4 (gamma ray series) measured by the macroscopic method, using the Hilger Spekker photometer with sections 48μ in thickness.

The form and position of the short wave-length absorption band near 2600 Å correspond to the adenine chromophor (cf. Holiday, 1930 ; Myrbäck, Euler and Hellström, 1932; Heyroth and Loofbourow, 1934*). (The presence

* Also unpublished measurements by Dr. C. B. Allsopp on yeast adenylic acid and by myself on muscle adenylic acid.

of uracil or of small amounts of guanine cannot be excluded.) At the wave-length 2537 Å the observed values of $\Delta_{uv} = 0{\cdot}89$ for Case 3 (X-ray series) corresponds to a concentration of yeast adenylic acid, 5·6 per cent. for a layer 6μ in thickness. This value is exceptionally high ; e.g. Case 1 (gamma ray

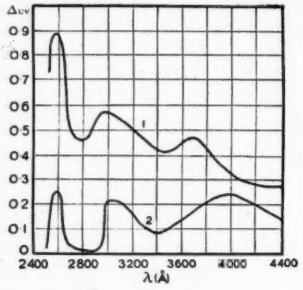

FIG. 18.—Approximate absorption spectrum of substances accumulating after X-irradiation. Case 3. Large spindle-cell sarcoma of antrum. Sections : Control and 80 min. after 516 r in 40 min. (Nominal thickness 6μ.)
Curve 1. Absorption spectrum of accumulating substances.
„ 2. Absorption spectrum of accumulating substances after exposure of fixed sections to ultraviolet radiation of wave-length 2537 Å for 575 hr.

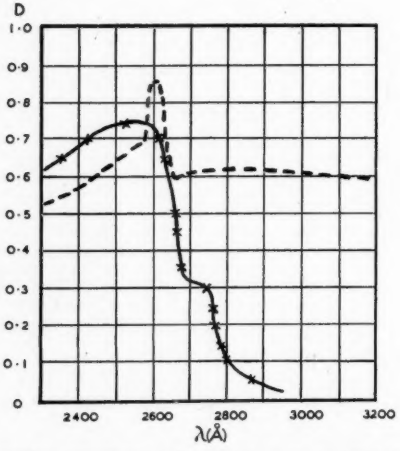

FIG. 19.—Difference absorption spectrum of control and irradiated sections measured by macroscopic method. (Sections 48μ thickness.)
— — — — Case 1. Control and 80 min. after 248 r gamma radiation in 4·45 hr.
×———× Case 4. Control and 80 min. after 388 r gamma radiation in 6·42 hr.

series) gave a value 1·8 per cent. The chromophoric group responsible for the absorption at longer wave-lengths has not yet been identified.

It is evident from Figs. 18 and 19 that the absorption spectrum of the substances accumulating after X and gamma irradiation may differ significantly in some cases from that described by Loofbourow, Dwyer and Lane

(1940) as characteristic of the proliferation promoting " inter-cellular hormones " produced by ultraviolet irradiation of living cells.

It is probable that the two absorption bands with maxima at approximately 3020 Å and 3670 Å are due to a different chromophoric group from that responsible for the band at 2580 Å, as shown by selective photochemical decomposition (Fig. 18, curve 2).

Fluorescence of Irradiated Tissues.

A small increase in fluorescence after X and gamma irradiation has been found, corresponding to the increase in ultraviolet absorption. The observed bluish-white fluorescence is of low intensity, but fluorescence microphotographs

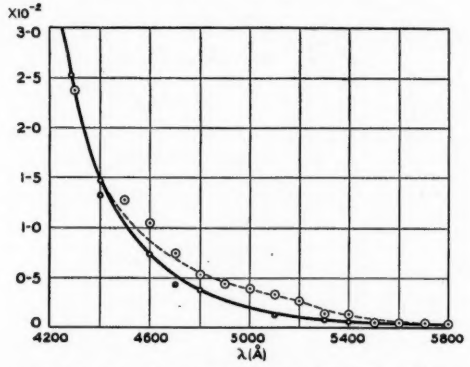

FIG. 20.—Case 1. Squamous-cell carcinoma of lip.
.—.—.—. Approximate spectral distribution of fluorescent radiation due to substances accumulating in tissue after gamma irradiation. Sections: Control and 80 min. after 248 r in 4.45 hr.
⊙—⊙—⊙ Approximate fluorescent spectrum of dried nucleoprotein layer. Wave-length of exciting radiation: 2950–4250 Å.

comparing the control and irradiated tissues have been obtained in a number of cases (1 and 4 of the gamma ray series ; 2 and 3 of the X-ray series). Figs. 16 and 17 (Case 1) are typical. No fluorescence was observed after the interposition of a layer of glass plates which excludes wave-lengths shorter than 3300 Å. Experiments with fresh unfixed biopsy specimens showed an apparently similar fluorescence which was not affected by fixation by Susa-alcohol or by heat. The microphotographs show that in epithelial structures the fluorescence is greatest in the basal-cell layer and adjacent parts of the stratum spinosum ; the keratinizing structures produce considerable scattering of radiation. The fluorescence does not differ significantly in intensity in the adjacent normal, hyperplastic and neoplastic squamous epithelial structures.

Quantitative measurement of the fluorescence spectrum of the substances accumulating after irradiation is difficult on account of the low intensity. An approximate method employing photographic photometry has been used, and in Fig. 20 is shown the spectral distribution of the fluorescent radiation due to the substances accumulating in a squamous-cell carcinoma of the lip

(Case 1) 80 min. after 248 r gamma radiation in 4·45 hr. ; in the same figure is included the approximate fluorescence spectrum of a dried layer of thymus nucleo-protein. In these experiments the wave-length of the exciting radiation was 2950–4250 Å.

Histochemical Evidence for the Presence of Pentose in the Cytoplasm after Irradiation.

The reaction with aniline acetate and concentrated hydrochloric acid applied as a histochemical test for pentoses is almost invariably positive in the cytoplasm of irradiated cells showing increased ultraviolet absorption at 2537 Å. Bial's orcinol reaction has been found to be less sensitive, but it is positive in specimens showing strongly positive aniline acetate reactions. The colours given by both the aniline acetate and orcinol reactions were transient and only persisted for several minutes. Irradiated sections showing increase in ultraviolet absorption also showed positive colour reactions with alpha-naphthol and concentrated sulphuric acid, and with phloroglucinol and concentrated hydrochloric acid, but with these reactions the sections were not satisfactory for microscopical examination. In normal, hyperplastic and neoplastic keratinizing squamous epithelial structures after X and gamma irradiation the positive pentose reactions are given by the cytoplasm of the basal cells and of many of the spinous cells, but with much less intensity by the cells of the stratum spinosum immediately beneath the stratum granulosum. In a basal-cell carcinoma of the skin (Case 8 of the X-ray series) there was only a slight increase after irradiation in the faint pentose reactions given by the cytoplasm of the tumour cells. The most strongly positive pentose reactions observed after irradiation were given by the cytoplasm of the cells of a large spindle-cell sarcoma (Case 3 of the X-ray series).

In *unirradiated* epithelial structures—normal, hyperplastic and neoplastic —faintly positive pentose reactions are often observed in the cytoplasm of cells of the basal layer and of the adjacent parts of the stratum spinosum. This result is in agreement with the findings of Caspersson (1941) and Caspersson and Schultze (1938, 1939). In both the control and irradiated sections pentose reactions are shown by a large number of the nucleoli ; as a rule there is no other nuclear staining, in agreement with the well-known failure of desoxyribose to give strong microchemical reactions for pentoses.

It is important to note that in no instance was a positive Feulgen reaction shown by cytoplasmic structures in control or irradiated tissues, so that the presence of desoxypentoses in the cytoplasm can be excluded.

Histochemical Evidence for the Presence of Purine and Pyrimidine Derivatives in the Cytoplasm after Irradiation.

The diazo reaction with diazotized sulphanilic acid (cf. Clara, 1934) failed to show any convincing difference between the control and irradiated sections. It was evident that the tyrosine and histidine residues of the proteins gave intense colour reactions which might mask any difference due to substances accumulating after irradiation. To avoid this difficulty, the sections were benzoylated before diazotization. The Schotten-Baumann reaction with

tyrosine is, of course, well known, and also it has long been recognized (cf. Kossel and Edlbacher, 1915; Ashley and Harington 1930) that attempted complete benzoylation of histidine and histamine leads to breaking of the iminazol ring. The diazo derivatives of many purine and pyrimidine compounds are well known (cf. Burian, 1901, 1904; Steudel, 1906; H. Fischer, 1909), but the diazo reactions of the corresponding benzoyl derivatives do not appear to have been described. Subsidiary experiments showed that after benzoylation adenine and uracil give orange-yellow colours, while guanine gives an intense crimson colour reaction, with diazotized sulphanilic acid followed by the addition of alkali.

The benzoylation of the sections by means of benzoyl chloride in the presence of aqueous NaOH requires great care and is best carried out at room temperature.

Method.

The control and irradiated sections are mounted side by side on a glass slide. After removal of the paraffin wax, a few drops of an approximately 2N aqueous NaOH solution are added and then a few drops of benzoyl chloride. A covers lip is placed on the sections, which are left at room temperature for 1–6 or even 12 hours. At the end of this time concentrated HCl is run underneath the cover slip, which floats off. The slide is washed with distilled water, then with 80 per cent. alcohol and finally with absolute alcohol.

The diazotization is carried out using Ehrlich's diazo reagent-diazotized sulphanilic acid (cf. e.g. Hutchison and Hunter, 1935, p. 250). Gentle warming is advisable. The diazo reagent is poured off and one spot of 2N NaOH added. Then, after several minutes the sections are washed in 80 per cent. alcohol, absolute alcohol and xylol and mounted in canada balsam.

In a number of the irradiated specimens showing increased ultraviolet absorption and positive pentose reactions, the diazo reaction after benzoylation showed a quite definite brownish-red staining of the cytoplasm of the basal and many of the spinous cells in normal and hyperplastic epithelium and in squamous-cell carcinomata. Only a faint yellow colour was shown by the epithelial structures on the control side, where in some cases the reaction was greatest in the basal cells. In the case of the specimens of the large spindle-cell sarcoma examined (Case 3), on the control side the tumour cells showed only a faint yellow cytoplasmic staining, but after irradiation there was usually a brownish-red cytoplasmic staining of the tumour cells. In no instance was there intense nuclear staining in either the control or irradiated sections, although many of the nucleoli in both the sections show brown staining. Thus, the diazo reactions after benzoylation are consistent with the presence in the cytoplasm of the irradiated cells of adenine, small amounts of guanine and perhaps also uracil.

As supplementary reactions, Hunter's diazo reaction (Hunter, 1936) in the presence of hydroxylamine and Na_2CO_3 invariably gave yellow but never red colour reactions; this finding makes the presence of thymine unlikely.

The histochemical application of the murexide reaction is notoriously difficult; negative murexide reactions were invariably given by both the control and irradiated sections. This finding would, of course, be consistent

with the presence of adenine, and also of alloxazine (Kuhn and Bär, 1934), but a negative murexide reaction cannot be regarded as very significant. Weidel's reaction (cf. Strauss and Koulén, 1929; Winterstein and Somló, 1933) is invariably negative; also Wheeler and Johnson's reaction is negative.

The presence of a positive diazo reaction after benzoylation in the cytoplasm of irradiated cells runs parallel with the finding of positive pentose reactions. The specimens from the following cases have been examined: Nos. 1, 4, 13, 15 of gamma ray series and 2, 3 of X-ray series.

Interpretation of the Increase in Ultraviolet Absorption of the Cytoplasm after X and Gamma Irradiation.

The observed increase in the optical density of the section of tissue which has received X or gamma radiation *in vivo* is of the order of 0·15 at the wave-length 2537 Å for a radiation dose of the order of 500 r, but may be considerably greater. These measurements refer to sections of thickness approximately 2μ; the absolute thickness of the sections is not known with precision, but with all the sections measured the error introduced by the difference in thickness of the sections is negligible in comparison with the observed absorption changes. The photometric measurements of the ultraviolet photomicrographs all refer to the wave-length 2537 Å, so that for their interpretation it is essential to utilize the additional information provided by the measurements of the ultraviolet absorption spectra and the histochemical tests. These experimental findings are consistent with the accumulation in the cytoplasm of proliferating and incompletely differentiated cells of a *pentose* (which is not desoxyribose) and purine (and pyrimidine) derivatives, probably *adenine*, and possibly also guanine in small amounts and uracil. The ultraviolet absorption spectrum of the accumulating substances cannot give any information concerning the pentose groups (cf. Holiday, 1930; Goos, Schlubach, Schröter, 1930). Further, the existence of the long wave-length region of ultraviolet absorption suggests that other chromophoric groups are present, and such groups may of course contribute to the absorption band near 2600 Å. The solubility properties suggest that the accumulating substances are nucleotides or nucleosides.

The increase in cytoplasmic density of 0·15 at the wave-length 2537 Å, with sections 2μ in thickness, can be accounted for by the accumulation of yeast adenylic acid in concentration 2·8 per cent. or phytonucleic acid in concentration 3·5 per cent. These values of course refer to the cytoplasm of the cells of the proliferating and differentiating areas only. The mean concentration for the whole tissue is of much less significance for the present work, because it depends mainly on the proportion of fully differentiated cells present together with the adult connective tissue and blood vessels of the stroma. The macroscopic method of measurement of the absorption spectrum determines the approximate lower limit of the mean concentration for the whole section, and in Case 3, for example, this value is approximately one-tenth of that obtained for the growing areas by the spectroscopic image method.

The possibility that the observed increase in absorption at 2537 Å could be attributed to "photochemical" changes in protein appears to be excluded. The observed changes in the absorption spectrum are quite unlike any of the

known changes produced either by ultraviolet radiation or by very large doses of X or gamma radiation; further, an increase in density of 0·15 at 2537 Å would require for its explanation an additional concentration or absorption increase of the proteins of the order of 200–700 per cent., and values twice as high as this would be called for to account for some of the experimental results. Quite apart from the improbability of such assumptions, it is evident from the work of Sanigar, Krejci and Kraemer (1939) that no detectable changes would result from photochemical changes produced in the cell proteins *in vitro* by the doses of X and gamma radiations used.

The most striking result of practically all the experiments reported in the literature is the insensitivity of the cell constituents *in vitro* to X and gamma radiations; almost invariably, detectable chemical changes are found only with necrotic doses (Arnow, 1936; Holmes, 1939); e.g. Jacobson (1939) could detect no chemical changes in a pterine of physiological importance after 1,000,000 r gamma radiation. However, in a few cases significant effects have been detected with doses within the therapeutic range, e.g. inhibition of glycolysis after irradiation at low temperature (Crabtree and Gray, 1939), inactivation of purified enzymes (Dale, 1940).

In a few instances the number of molecules transformed per ion pair has been measured, e.g. for tyrosine in solution the value is approximately $\phi = \frac{1}{12}$ (Stenström and Lohmann, 1928); of particular interest are the results of Gunther and Holzapfel (1939), who found that water vapour and liquid water (but not ice at approximately −180° C.) are decomposed by X radiation with production of 1 H_2 molecule per ion pair (cf. Fricke, 1935).

As a preliminary to the present work a series of experiments (unpublished) have been carried out in collaboration with Dr. C. B. Allsopp on changes in the ultraviolet absorption spectrum of compounds of physiological interest after X and gamma irradiation. Although not completed, these experiments have given maximum values for the number of molecules transformed per ion pair; the results are for yeast adenylic acid 9, glycyl-tyrosine 11, the tyrosine groups of insulin 27, and indol 18. These are preliminary values, and it appears probable the true values may be much lower in some cases. However, these experiments provided data for calculating the order of the changes to be expected in the histological preparations as a result of purely photochemical changes due to the X and gamma radiation without the intervention of "physiological" processes. In a 2μ layer of a 10 per cent. solution of yeast adenylic acid and for a dose of 1000 r assuming that the number of molecules decomposed per ion pair $\phi = 10$, the optical density would decrease by approximately 10^{-4}; for a density change of the order of 0·1 (which in this case would be a *decrease* in contrast with the observed increase) ϕ would have to be of the order 10^4. Similarly for a layer 2μ in thickness of a 20 per cent. solution of a protein containing 6 per cent. tyrosine and 2 per cent. tryptophane, and assuming that the chromophor of tryptophane behaves like indol, irradiation with a 1000 r X or gamma radiation would produce a decrease in density of the order of 10^{-5}; this result is consistent with the experimental findings of Sanigar, Krejci and Kraemer (1939). It can be concluded that even if X and gamma radiations produce photochemical changes with an efficiency as

high as 10 molecules transformed per ion pair, no changes in the ultraviolet absorption of the histological preparations could be detected with a dose of 1000 r. (All the above calculations refer to the wave-length 2537 Å.)

Changes in the optical density of the magnitude observed in the ultraviolet photomicrographic measurements imply that either "photochemical" processes are occurring with extremely high efficiencies of the order 10^3–10^4 molecules transformed per ion pair, or that the absorption changes are due to complex secondary "physiological" processes resulting from photochemical changes occurring with normal efficiencies of the order of unity. Changes of very high efficiency may be explicable in terms of a chain reaction mechanism, and suggest the possibility of enzyme inhibition by the radiation, but the necessary experimental evidence is not yet available.

SUMMARY.

An increase in the absorption of ultraviolet radiation of wave-length 2537 Å in the cytoplasm of proliferating and differentiating cells has been found after therapeutic doses of X and gamma radiations. The dependence of this absorption increase upon dose and dose rate and the influence of recovery processes are discussed. As yet no significant difference in absorption increase has been observed between normal and hyperplastic tissues and malignant tumours.

The increase in ultraviolet absorption of the cytoplasm after irradiation is shown to be due to the accumulation of pentose nucleotides, probably ribonucleotides, containing adenine and some other unidentified chromophoric groups. The magnitude of the change is consistent with the presence in the irradiated cytoplasm of ribonucleotides in local concentration often of the order of 3 per cent., and suggests the production of a metabolic disturbance by the radiation.

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DISTURBANCE OF NUCLEIC ACID METABOLISM PRODUCED BY THERAPEUTIC DOSES OF X AND GAMMA RADIATIONS. PART III: INHIBITION OF SYNTHESIS OF THYMONUCLEIC ACID BY RADIATION.

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Nucleic Acid Content of the Nuclei after Irradiation.

Absolute measurements of the amount of nucleic acid in individual nuclei have been described by Caspersson (1936, 1940). Similar measurements were not possible with the apparatus available, and it has been necessary to employ photographic photometry for measurements of possible changes in absorption occurring in the nuclei after X and gamma irradiation. These measurements give quantitative information concerning the concentration of purine and pyrimidine groups present, but cannot detect changes in the pentose or phosphate groups (cf. Holiday, 1930; Goos, Schlubach and Schröter, 1930). The "nucleic acid" estimated thus includes both ribo- and desoxyribo-nucleic acids and the corresponding nucleotides, nucleosides and bases. Three methods have been employed:

I. *Measurement of mean changes in absorption and size per nucleus.*

Measurements have been made of the density of absorbing materials within the nuclei of the cells of the control and irradiated sections in areas of strictly comparable and carefully selected histological structure. Measurements of the corresponding nuclear diameters were also made in addition to measurements of the absorption of the adjacent cytoplasm.

This method is difficult to apply because of the inhomogeneity of the

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structure of many of the fixed nuclei. Among the plates examined with the non-recording microphotometer, using the methods described in Part II, in only one instance (Case 8 of the gamma ray series) was it possible to make really satisfactory measurements on a sufficient number of nuclei. The area selected was the stratum spinosum of the hyperplastic keratinizing squamous epithelium adjacent to a basal-cell carcinoma. The measurements of the nuclei showed no significant change in density after irradiation. The plates were measured twice and in each case for the nuclear contents $\Delta_{uv} = -0{\cdot}01$; statistical analysis of the observations gave for $\nu = 19$, $t = 0{\cdot}38^3$, $P_s = 0{\cdot}647$ and $t = 0{\cdot}91^2$, $P_s = 0{\cdot}813$ for the two sets of readings. For the geometric mean nuclear diameters measured at magnification $\times$ 670 the values were $4{\cdot}6^0$ mm. on the control side and $4{\cdot}4^4$ mm. on the irradiated side. This difference was also not significant ($\nu = 19$, $t = 0{\cdot}61$, $P_s = 0{\cdot}722$). Thus it can be concluded that in this case there was no significant change in the nucleic acid content per nucleus 80 min. after 575 r gamma radiation given continuously in 18·0 hr.; however, measurements of the absorption of the adjacent cytoplasm showed a significant increase after irradiation, $\Delta_{uv} = 0{\cdot}07$; for $\nu = 18$, $t = 7{\cdot}2$, $P_s = 1{\cdot}000$.

II. *Changes in density of individual nuclei measured with recording microphotometer.*

Measurements of the equivalent visible density of the nuclei and cytoplasm at points along the photometer tracings have been made in a number of cases and are summarized in the table. (In all cases tracings were made of the image of the standard neutral wedge.) The method is limited practically to the examination of relatively small numbers of nuclei and depends on the selection of histologically comparable cells. It has the advantage of considerably greater resolving power than that available for the individual measurements in method (1); the recording microphotometer had a slit of effective width 0·01 mm. and length 0·5 mm. In the table the "nuclear margin" includes the nuclear membrane and the peripheral chromatin of the fixed nucleus. This method can only give information concerning changes in the concentration of chromophoric groups in the nuclear margin and in the adjacent cytoplasm. The results given in the table, which includes only measurements on satisfactorily comparable histological structures, shows that the density increase in the nuclear margin is invariably much less than that

TABLE I.—*Measurement of Optical Density of Nuclei and Cytoplasm from Photometer Records.*

		Equivalent visible density.					Δ_{vis}.		Δ_{uv}.	
		Control.			Irradiated.					
Case.		Cytoplasm.	Nuclear margin.	Blank.	Cytoplasm.	Nuclear margin.	Cytoplasm.	Nuclear margin.	Cytoplasm.	Nuclear margin.
Gamma ray series	1	1·09	1·16	1·05	1·26	1·25	0·17	0·09	0·19	0·10
	7	1·69	1·75	—	1·76	1·78	0·07	0·03	0·08	0·03
	9	1·68	1·72	—	1·82	1·79	0·14	0·07	0·16	0·08
X-ray series	3(i)	1·48	1·48	1·46	1·80	1·73	0·32	0·25	0·37	0·29
	3(ii)	1·28	1·31	1·17	1·36	1·31	0·08	0·00	0·09	0·00
	7(i)	2·13	2·17	1·99	2·23	2·20	0·10	0·03	0·11	0·03

in the cytoplasm. In three of the cases there is no significant change in the nucleus, while the adjacent cytoplasm shows a typical increase in absorption. All these measurements of course refer to the wave-length 2537 Å.

III. *Comparison of the increase in total absorption with that of the cytoplasm.*

In some instances it has been possible to compare the increase in optical density of small homogeneous areas of the sections at 2537 Å, as measured by the " spectroscopic image " method, with the mean increase in cytoplasmic absorption measured microphotographically on representative fields within the same area. For example, in Case 1—squamous-cell carcinoma of lip—at 2537 Å the mean increase in density for the selected small area of the whole section of nominal thickness 6μ was found to be 0·27 ; the mean increase in cytoplasmic absorption for sections of nominal thickness 2μ was $\Delta_{uv} = 0{\cdot}15$. Measurements of the latter fields show that approximately 73 per cent. of the total selected area is occupied by cytoplasm, so that the mean increase in density of the whole section due to the cytoplasmic change alone would be 0·11, which is in agreement with the mean value 0·09 for the whole section of thickness 2μ deduced from the direct measurements on the sections of thickness 6μ. The closeness of the agreement is probably fortuitous in view of the errors involved, but it can be concluded that in this case there is no significant change in the mean nucleic acid per nucleus after irradiation.

From these measurements made by the three different methods it can be concluded that at 80 minutes after irradiation the nucleic acid content of the nuclei shows either no significant change or an increase corresponding to a change in concentration of total thymo- and phyto-nucleic acid considerably smaller than that shown by the adjacent cytoplasm. The inclusion in the nuclear " margin " of a " rim " of overlapping cytoplasm may account in some cases for the apparent nuclear changes, and diffusion during fixation cannot be excluded. The time, 80 min., at which the biopsy specimens were taken after the end of irradiation was selected to allow the cells caught in division at the time of exposure to complete mitosis. The mitotic inhibition in the specimens examined has been confirmed by cell counts kindly carried out by Dr. A. Glücksmann in the course of his investigation (cf. Glücksmann, 1941).

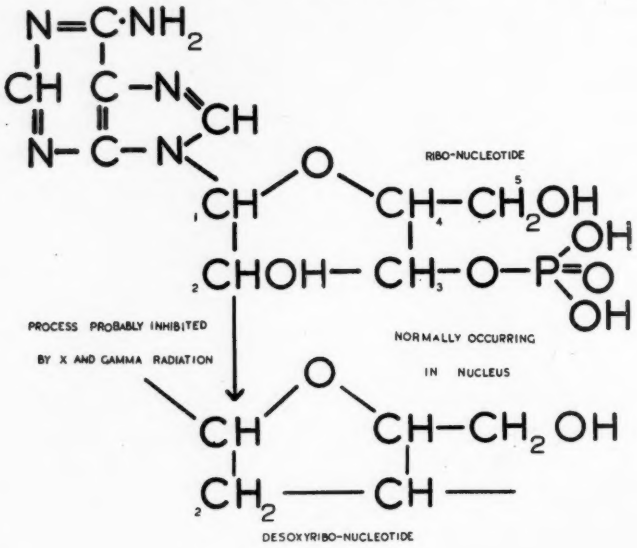
GENERAL DISCUSSION.

The experimental evidence discussed in Part II shows that small doses of X and γ radiation produce in proliferating and differentiating cells a disturbance of nucleic acid metabolism, of which one aspect is the accumulation in the cytoplasm of the affected cells of pentose nucleotides, in high concentration, often of the order of 3 per cent. The pentose is probably ribose, but never desoxyribose, and the ultraviolet absorption spectra in conjunction with the histochemical tests suggest the presence of adenine and possibly guanine and uracil, together with some other identified chromophoric groups. The cytoplasmic ribonucleotides thus include substances similar chemically to the nucleotides found normally in much lower concentration in the cytoplasm of many rapidly growing cells, and can reasonably be assumed to accumulate as a result of a disturbance of the normal metabolic processes either by

increased rate of formation or decreased rate of removal. The striking increase of ribonucleotides in the cytoplasm of individual cells after irradiation leads to a corresponding increase in the total amount of nucleotides per unit volume of tissue.

At the same time there is no comparable increase in the nucleic acid content of the nuclei after irradiation ; often there is no significant change in the mean nucleic acid content per nucleus at 80 min. after irradiation when mitosis shows maximum inhibition. Any observed nuclear change of course includes both thymo- and phyto-nucleotides, since the carbohydrate groups make no significant contribution to the total light absorption.

A probable explanation of these quantitative experimental results is that



X and γ irradiation inhibits the normal synthesis of thymonucleic acid by stopping the reduction of ribonucleotides to desoxyribo-nucleotides (see Fig.). The mechanism of this process is not known, but the work of Caspersson (1941) suggests that the final stages of the synthesis of thymonucleic acid take place within the nucleus. The accumulation of ribo-nucleotides in the cytoplasm can be regarded as due to the greatly increased stationary concentration resulting from inhibition of the normal processes of removal. The appearance of ribonucleotides in the cytoplasm of proliferating and differentiating cells with no obvious relation to mitosis is consistent with the work of Marshak (1941) ; the possibility of an increased rate of production of ribo-nucleotides after irradiation cannot be excluded. It is consistent with the known experimental facts concerning the action of radiation on cells that the failure to synthesize thymo-nucleic acid after irradiation is due to changes produced within the nucleus or perhaps in the nuclear membrane. The accumulation

of nucleotides in the cytoplasm of irradiated cells thus does not necessarily imply a direct action of radiation on the cytoplasm.

The role of phosphate in the metabolic changes following irradiation needs detailed investigation. Marshak (1941) has shown by means of radio-active phosphorus (P^{32}) that after a small dose (200 r) of X radiation there is a large change in the distribution of total P^{32} in the cells of a lymphoma; the concentration of phosphorus increases in the nuclei and decreases in the cytoplasm at 2 hr. after irradiation, when mitotic inhibition was greatest. In order to correlate these findings with the present work one may envisage the possibility that adenosine triphosphate synthesized in the cytoplasm is normally transported into the nucleus and dephosphorylated—perhaps by transfer of phosphate to the arginine and lysine residues of histones. After irradiation the adenine riboside liberated can no longer be reduced to desoxyriboside and utilized to form chromosomes, but diffuses out of the nucleus into the cytoplasm.

The present experimental results appear to offer a simple explanation of the histological changes observed after X and γ irradiation. Inhibition of the synthesis of thymonucleic acid provides a mechanism for the well-known inhibition of mitosis. The accumulation of ribo-nucleotides in the cytoplasm may possibly account for the main cytoplasmic changes after irradiation, viz., differentiation and increased volume, in terms of the conception advanced by Caspersson (1941) that ribo-nucleotides are in some way responsible and essential for protein synthesis.

SUMMARY.

X and gamma radiations in therapeutic doses produce in proliferating and incompletely differentiated cells a disturbance of nucleic acid metabolism characterized by the inhibition of the synthesis of thymonucleic acid in the nucleus and the accumulation of ribo-nucleotides in the cytoplasm of the affected cell. The changes are probably due to inhibition by the radiation of the process of reduction of ribo-nucleotides to desoxyribo-nucleotides in the nucleus. Such a mechanism appears to be consistent with the histological changes observed after irradiation.

Finally, I wish to thank Prof. J. A. Ryle for providing facilities for this work, Dr. A. W. Calm for his help in carrying out many of the experiments, Mr. E. W. Mitchell for his willing technical assistance, Dr. F. G. Spear and Dr. A. Glücksmann for many discussions and invaluable help on the histological side, and Dr. E. Friedmann for his helpful criticism; further, I gratefully acknowledge the financial assistance of the British Empire Cancer Campaign and of the Sir Halley Stewart Trust.

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A PRELIMINARY DESCRIPTION OF PREPARATIONS OF SOME OF THE VIRUSES CAUSING TOBACCO NECROSIS.

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WITH AN ADDENDUM ON

EXAMINATION IN THE ULTRACENTRIFUGE.

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TOBACCO necrosis was named and described by Smith and Bald (1935); they showed that it differed in two main ways from any previously recognized plant virus disease. Firstly, no host was found in which systemic infection occurred, and secondly, the symptoms on naturally infected tobacco plants differed from those on plants inoculated experimentally. Symptoms appeared only on the lowest leaves of naturally infected plants and consisted of a necrosis spreading from and along the veins, while the upper leaves looked healthy and were virus-free. Nevertheless, these leaves were not immune, for when rubbed with infective sap they developed lesions. The lesions were restricted to the leaves actually rubbed and consisted of discrete necrotic rings or spots. Plants other than tobacco, such as cowpea (*Vigna sinensis*) and French bean (*Phaseolus vulgaris*), were also found to be susceptible and to give necrotic local lesions when inoculated. Smith (1937) found that the roots of tobacco and of some other plants were often infected, although neither the roots nor the leaves showed any symptoms; extracts of the roots of such apparently healthy plants produced the typical necrotic lesions when rubbed on to leaves of bean or tobacco.

Pirie *et al.* (1938) purified Smith's culture of tobacco necrosis virus with results different from any obtained with other viruses. Their end-product invariably consisted of two fractions, one of which crystallized in the form of thin plates, whereas the other was amorphous. The crystalline and amorphous fractions gave different sedimentation constants. Bawden (1941) showed that the symptoms typical of tobacco necrosis could be caused by serologically unrelated viruses. He purified two cultures of serologically unrelated tobacco necrosis viruses and got no crystals, but showed that the culture used by Pirie *et al.* (1938) contained both of these, and suggested that the crystalline

fraction of their products was yet another virus serologically unrelated to the amorphous ones. We have now confirmed this suggestion. We have purified tobacco necrosis viruses from other sources and obtained crystals, some in the form of flat plates and others in forms previously undescribed. This paper consists largely of a description of the preparation of these crystals and of the serological reactions of the different viruses.

MATERIALS AND METHODS.

Tobacco necrosis viruses from four different sources have been used. The first was derived from a single local lesion on a tobacco leaf, produced by rubbing with an extract from the roots of an apparently healthy potato plant. The second came from the roots of an apparently healthy tobacco plant. The third source was a single local lesion on bean, produced by using as inoculum a dried, necrotic bean leaf supplied by Dr. W. C. Price, of Princeton, U.S.A. The fourth was a direct descendant by bulk culture from that previously described as the Rothamsted culture (Bawden, 1941). These four cultures will be referred to as the potato, tobacco, Princeton and Rothamsted cultures respectively.

The first and third were derived from single lesions ; it is therefore reasonable, in the absence of any evidence to the contrary, to assume that they consist of only one strain of virus.

It became obvious, after several separate preparations had been carried through, that the second culture was a mixture. Bean leaves were therefore rubbed with diluted infective sap and six discrete lesions were isolated and used as inocula on six separate bean leaves. Sap from these leaves gave enough inoculum for preparations from tobacco plants. These isolates are called tobacco I to VI. The Rothamsted culture, being a bulk culture, may well be a mixture, but we have made no attempt to prove this. This set of names has arisen purely as a matter of convenience during the investigation, and each name refers only to the origin of the culture and has no systematic connotation.

Six antisera have been used. Two were prepared by Dr. E. T. C. Spooner in 1938 ; one of these was the serum used in the work of Pirie *et al.* (1938), and the other was prepared against the crystalline fraction of their product, but had not been tested before. The other four were prepared by Dr. A. Kleczkowski against the Rothamsted and potato cultures and against two of the single lesion isolates from the tobacco culture. All the virus preparations were good antigens, and antisera precipitating at dilutions greater than 1 in 200 were made by two injections of 0·5 mg. of purified virus into rabbits. The tests were made as previously described (Bawden, 1941).

We have tried many methods of purifying these tobacco necrosis viruses, but have found none that is uniformly satisfactory. Except for the Rothamsted culture, which is much less resistant to the purification processes than the others, the following method has given the best results, although sometimes only one-quarter of the virus present in the original sap is recovered in the final product. No doubt better methods can be devised using a quantity high-speed centrifuge, but we are not yet in a position to use this method.

Leaves are picked from 6–8 days after inoculation, when the lesions are

well developed, put through a domestic meat mincer, and the mince frozen solid. This freezing is not essential, but it tends to increase the yield and gives a product that is easy to handle. After thawing, the sap is expressed by hand through thin, closely woven cloth, such as madapollam, and the leaf residue is mixed with a 4 per cent. solution of Na_2HPO_4. If tobacco leaves are used 250 ml. are added for each litre of sap, but with bean leaves it is well to use 360 ml. Phosphate precipitates much of the normal leaf protein and calcium ions, and the partial removal of these at this stage lessens the loss of virus by adsorption that their removal at a later stage entails. The success of the later alcohol precipitation does, however, depend on the presence of some calcium, for no virus can be separated by this method from sap to which an excess of potassium oxalate has been added. The precipitate of calcium phosphate has also the virtue of adsorbing some coloured contaminants that are otherwise difficult to remove from the preparation. The residue is again minced, the liquid is expressed, mixed with the sap, and half a volume of 90 per cent. alcohol is added with continuous, thorough stirring. The green coagulum is centrifuged off and discarded. Approximately 2 N HCl is added to the fluid to bring the pH to 4·5 or 4, and this protein precipitate is also centrifuged off and discarded. Two litres of 95 per cent. alcohol are added to each litre of centrifugate and the mixture is stirred occasionally for an hour; after lying for another hour, as much of the fluid as possible is removed by decantation. The precipitate contains the virus and is packed tightly by centrifuging at 3500 r.p.m. for half an hour. It is extracted with a 3 per cent. solution of ammonium sulphate in 0·5 per cent. acetic acid; the calcium salts, of which the precipitate is largely composed, are converted into hydrated calcium sulphate and the virus goes into solution. Three extractions are generally adequate if the mixture is left for about an hour during each extraction before centrifuging, and if the combined extracts have about one-tenth the volume of the original sap. Saturated ammonium sulphate solution is added to the clear brown extract at the rate of 30 to 60 ml. per 100 ml. The smaller volume is sufficient for tobacco VI virus, but the large volume is safer with the others. In general there is no immediate precipitate, for most of the normal leaf protein has been removed in the earlier stages. Even after 5 hours the precipitate may be barely visible; after 5 to 24 hours at room temperature the fluid is centrifuged for at least 45 minutes at 3500 r.p.m. If the removal of protein at the earlier stages has been satisfactory, a litre of sap will give 1 to 2 ml. of clear and nearly colourless precipitate. This is extracted two or three times with water so that the final volume of extract is about 1/100 of that of the sap. The combined extract is colourless and faintly opalescent, and should show no anisotropy of flow when shaken between crossed polarizers. The insoluble residue is either colourless and of a heavy, coarsely crystalline texture (presumably calcium sulphate) or is darkly coloured. If it is pale and settles out of solution slowly after stirring, it probably still contains virus. This can be recovered by extracting for 4–5 hours at 0° C. with 2 per cent. sodium chloride solution. The slowness with which crystalline virus precipitates sometimes dissolve will be commented on later.

With all the cultures this method usually leads to a loss of virus on the discarded precipitates. If the products from the potato, tobacco and Princeton

cultures are diluted to give the same serological titre as the original sap, however, they are as infective as the sap and often more so. Price and Wyckoff (1939) have also commented on a similar increase in infectivity with their preparations made by ultra-centrifugation. The reason for this increase is not clear, but it is most likely that purification removes material that acts as an inhibitor of infectivity. Such phenomena make it impossible to be certain what proportion of the initial virus is isolated in a fully active form.

The Rothamsted culture gives very variable results with this method. Occasionally it yields an infective product, but most often the product is serologically active, but almost non-infective. As a rule the infectivity is lost during the precipitation with alcohol, but it may survive this and still be lost in later operations. Using the method described by Bawden (1941) we have sometimes got reasonably active preparations of this culture, but even this usually leads to a product that is much less infective than that from the other cultures (Table I), and less infective than the virus in freshly-expressed sap. This method gives satisfactory products with the other strains and, when a large volume of sap is being handled, is to be recommended ; on a small scale, however, the method described here is both more convenient and more reliable.

The virus from all four cultures is more soluble in dilute ammonium sulphate solution at 0° C. than at room temperature, and this property can be used for fractionating the colourless products further and for their crystallization. Saturated ammonium sulphate solution is added drop by drop to the partially purified preparation at 20° C. until the first signs of opalescence appear, when it is cooled to 0° C. and left for a few hours. If a precipitate separates it is centrifuged off at 0° C., using the method of lagging a centrifuge tube that we have described (Bawden and Pirie, 1942). This precipitate is generally free from virus and can be discarded ; the supernatant fluid is kept at 0° C. for crystallization to proceed. Crystallization may begin in a few hours, but occasionally, for reasons that we do not understand, it is delayed for several weeks. If there is no crystallization in a few days a drop of saturated ammonium sulphate solution should be added for each ml. of fluid, but if more than a quarter of a volume of ammonium sulphate solution is added the precipitate that separates is likely to be amorphous.

Preparations of all cultures have been made from both beans and tobacco, and the host has not been found to influence the end-product. Purification of preparations from infected beans is generally the more difficult, for a larger amount of normal leaf protein remains in a water-soluble state in the fluid from which the virus is being crystallized. Unlike the viruses, this protein is more soluble at room temperature than at 0° C., and is largely removed when the preparation is centrifuged at 0° C. before crystallization. Further traces of normal protein can also be separated from dialysed preparations at 0° C. The somewhat erratic behaviour of these viruses on dialysis is described later in this paper.

YIELDS.

No definite figures can be given for the yields of virus, for these vary with the numbers of lesions on the leaves used for the preparation. In general,

however, the yield is higher from tobacco than from bean, and is higher when plants are grown under poor light conditions during the winter than in the summer. Purification is also considerably easier in the winter, for then the plants contain smaller quantities of other soluble proteins that interfere with the separation of the virus. Constant differences are obtained between the yields from different cultures. With tobacco I and II the yield from plants with large numbers of lesions varies from 1 to 6 mg. per litre, whereas with other cultures it varies from 15 to 100 mg. per litre. This difference is reflected in the infectivity and the serological titres of sap taken from leaves infected with the different cultures. Often sap with tobacco I and II gives no precipitate with antiserum, and the highest dilution ever to react was 1 in 4. By contrast, sap from plants rubbed with the potato, tobacco VI, Princeton and Rothamsted cultures precipitates with antiserum at dilutions of from 1 in 8 to 1 in 64 ; it is also 10 or more times as infective as sap from plants infected with tobacco I or II.

PROPERTIES.

Crystal Forms of the Different Viruses and Virus Strains.

All the viruses resemble Bushy Stunt in that they dissolve more slowly when in the crystalline state than when amorphous, and a useful fractionation can often be made by washing the crystals quickly with water. After crystallization it is often difficult to get the viruses completely into solution. They give opalescent suspensions that flocculate on warming and can give confusing results in serological tests. When left for some hours at 0° C., however, these suspensions become nearly clear and then remain stable on warming. This effect is probably responsible for at least part of the increase in clarity that was noticed by Pirie *et al.* (1938) as a result of freezing a mixture of tobacco necrosis viruses.

Potato culture.—This culture has behaved quite systematically, and the eighteen preparations that have been made have all crystallized completely, after lying at 0° for a few days, in the form of thin, lozenge-shaped plates (Fig. 1). The smaller pair of angles made by the edges of these plates is 79° ; this is the same as the angle in the crystalline material made by Pirie *et al.* (1938), and there seems to be no reason to think that the potato culture is not identical with the crystalline fraction of that preparation. Solutions rendered opalescent by the addition of ammonium sulphate have an intense sheen and show anisotropy of flow when shaken between crossed polarizers. The crystals are birefringent when viewed edgeways. Crystallization seems to proceed by the regular piling of these plates on one another as in a pack of cards, for preparations that are examined under the microscope after the minimum disturbance contain thicker plates than usual, and show but little anisotropy of flow when shaken lightly. On more intense shaking the plates separate and the usual amount of anisotropy of flow appears. Further evidence that this is the original manner of crystallization is given by the frequency with which an identical nick appears on many of the malformed crystals in a preparation. Slotta and Fraenkel-Conrat have described (1938) a somewhat similar manner of crystallization with the protein from rattlesnake venom.

Tobacco culture.—Preparations from this culture have always given crystals, but the crystal form has varied widely in successive preparations. Dodecahedra, bipyramids, irregular laminae and other forms were all encountered, and sometimes more than one form was present in the same preparation (Fig. 3). It seemed likely that these variations resulted from the fact that the culture contained a mixture of viruses. Attempts to avoid this difficulty were made by isolating six local lesions from this culture and using each as a separate inoculum, in the hope that a single local lesion would contain only one virus. Only three of the isolates have been studied in any detail; these will be referred to as tobacco I, II and VI. The preparations of VI have behaved quite consistently, whereas those of I and II have varied, although we have no reason to believe that they are mixed cultures.

The crystals separated from the first few preparations of I and II were dissimilar, so that it was thought that the cultures were probably different, but, after six separate preparations of each had been made, each had given all the different crystal forms. For these and other reasons, it now seems likely that I and II are very similar if not the same. Four modes of crystallization have been recognized. From solutions more acid than pH 5 the virus separates as irregular laminae; when these form on the walls of the vessel they have the more or less circular outline shown in Fig. 6 and they are generally slightly puckered. In the bulk of the fluid, pieces of irregularly crumpled sheet are to be seen. Between pH 5 and pH 7 the usual forms are bipyramids, dodecahedra and several types of twinned crystal. The bipyramids may be mistaken for regular octahedra when they are small, but when large crystals separate their birefringence is apparent and they tend to be elongated as shown in Figs. 9 and 10. The rhombic dodecahedra appear to be regular and even the largest have shown no birefringence. A variety of many-pointed stars has been found, but the most common type is shown in Fig. 4. It appears to be a cube on to each of the six faces of which a square pyramid has been built; the angle at the apex of the pyramid is, however, more acute than that which would have resulted in a rhombic dodecahedron, and a 24-faced solid is formed. These crystals often seem to have a laminated structure. Except that the irregular laminae are formed in acid solution, the conditions leading to the different manners of crystallizations are not understood. After clarifying the infective sap, we have sometimes split it into two and worked up the two parts separately, and from one half we have obtained rhombic dodecahedra and from the other birefringent bipyramids. Similarly, we have dissolved dodecahedra, re-crystallized and obtained bipyramids. Preparations at the stage for crystallization have been split, and part seeded with dodecahedra and part with bipyramids, but this has introduced no more consistency into the crystallization. Soon after the addition of ammonium sulphate solution all preparations of I and II show a definite sheen, but this disappears in a day or two when crystallization is complete.

Tobacco VI has always crystallized in the form of hexagonal prisms, with pointed ends. The only variation is that sometimes lateral growth proceeds so far that the points are replaced by hexagonal faces; this is illustrated in Figs. 5 and 7.

These prisms are birefringent when viewed from the side, but not when

viewed end on (Fig. 8). Crystallization is slower with this virus than with any of the others, and is often only complete after a fortnight. The difference in the solubility of this virus at 0° C. and 20° C. seems greater than that of the others, for the addition of sufficient salt to produce an opalescence at 20° C. is often insufficient to produce crystals when the solution is kept at 0° C.

Princeton culture.—After a short time at 0° C. in ammonium sulphate solution, purified preparations of this culture develop an intense sheen and, if shaken between crossed polarizers, they show strong anisotropy of flow. When examined microscopically the precipitate is seen to consist of small uniform particles, resembling bacteria about one-and-a-half times as long as they are wide. This elongation would seem insufficient to account for the pronounced optical phenomena and it is possible that the particles are plate-like. We have attempted to increase their size by slow precipitation methods, but have obtained nothing that can certainly be considered a crystal.

Rothamsted culture.—From a large number of preparations of this culture we have never obtained any crystalline material. Occasionally the preparations have given a sheen when precipitated with ammonium sulphate, but otherwise the precipitates have been amorphous. It is, of course, possible that the loss of infectivity suffered during purification is responsible for this, and that purified, fully-active preparations would crystallize. With other viruses, such as bushy stunt and the potato culture of tobacco necrosis, loss of infectivity without loss of serological activity has no effect on the ability to form crystals. Unless the changes undergone by the Rothamsted culture on purification are different from those rendering these other viruses non-infective, this explanation is unlikely to be true.

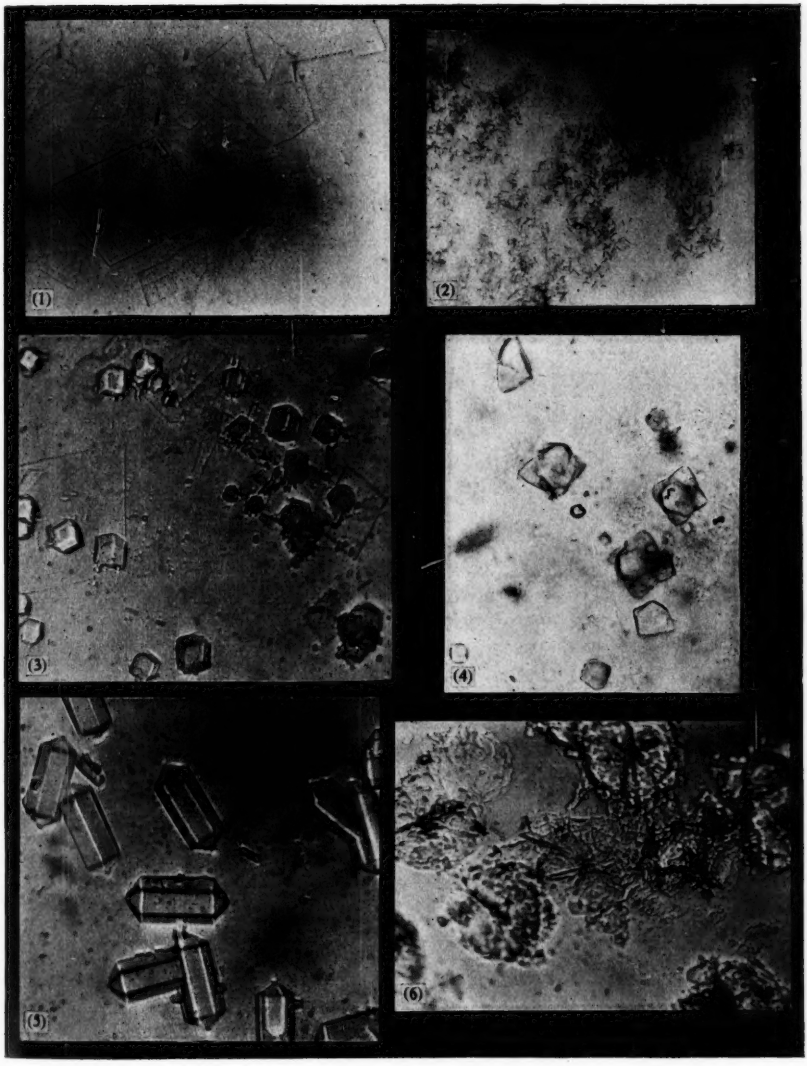
ACTIVITY AND SEROLOGICAL RELATIONSHIPS.

Table I shows the activity of a purified preparation of each culture. These tests were done at different times, so that exact comparisons of the infectivity of the different cultures cannot be made. The reduced infectivity of the purified preparation of the Rothamsted culture, however, is obvious, and often the effect is greater than the one shown. The higher serological titre is a further constant feature of preparations of this culture. This is probably

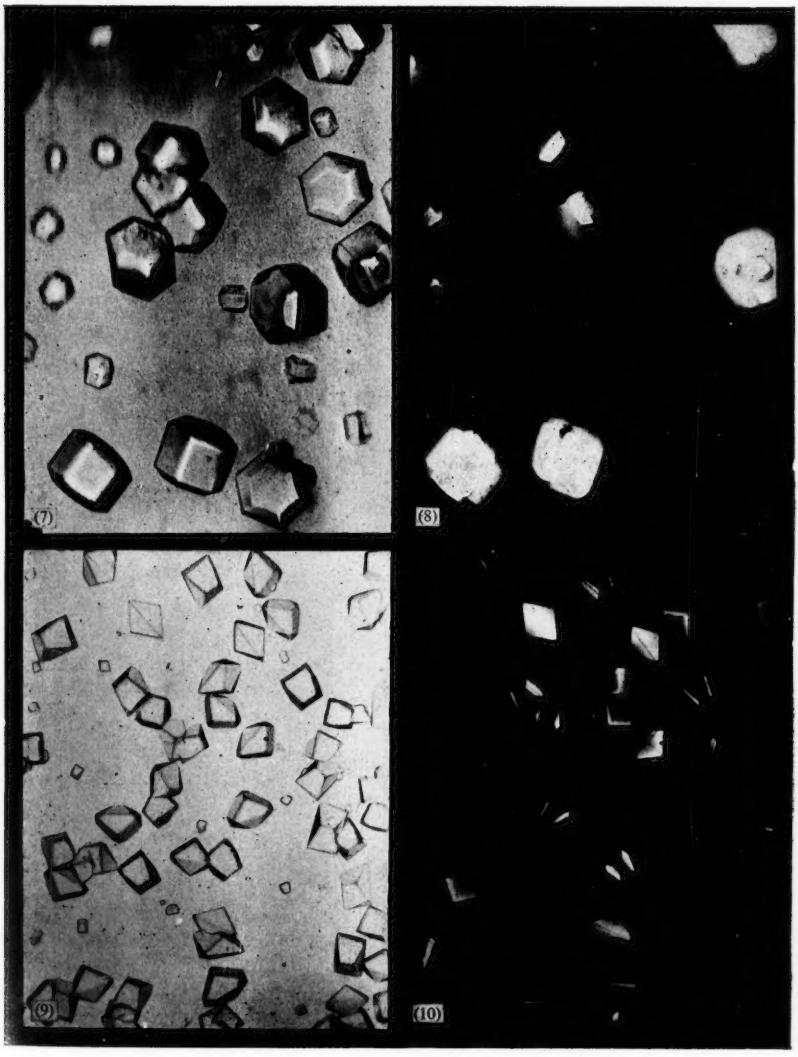
EXPLANATION OF FIGURES.

Crystalline forms of Tobacco Necrosis Viruses.

1.—Lozenge-shaped crystals from the potato culture. × 200.
2.—Crystals of the potato culture, some seen edgewise, photographed while still attached to the wall of the tube in which the preparation was crystallized. × 70.
3.—Crystals from the original tobacco culture before single lesion isolates were made. Lozenge-shaped crystals and dodecahedra occur together. × 70.
4.—Twinned crystals from a culture of tobacco I. × 200.
5.—Crystals in the form of hexagonal prisms with pointed ends, produced by rapid crystallization of tobacco VI. × 200.
6.—Crystals of tobacco I. Round laminae separating on the walls of the tube from a preparation at pH 4·5. × 200.
7.—Tobacco VI crystallized slowly. × 70.
8.—The same field as fig. 7, photographed between crossed Nicol prisms. × 70.
9.—Tobacco II in the form of bipyramids. × 70.
10.—The same field as fig. 9, photographed between crossed Nicol prisms. × 70.



Bawden and Pirie.



Bawden and Pirie.

another intrinsic difference between the virus (or viruses) in this culture and in the others, for the least rigorous purification methods are used with this culture ; it is therefore the most likely to contain impurities and its serological titre may be minimized in our tests.

TABLE I.—*Activity of Purified Preparations of Different Cultures of Tobacco Necrosis Viruses.*

Culture.	Serological titre.	Infectivity. Average number of lesions per leaf at—			
		10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.
Potato	1/400,000	350	115	23	3
Tobacco I	1/300,000	270	72	26	5
Tobacco VI	1/400,000	300	80	13	2·5
Princeton	1/400,000	325	120	20	2
Rothamsted	1/600,000	86	9	1	0

When sap from plants infected with any of the cultures is dried and resuspended, its infectivity and serological activity are not greatly affected. Drying after purification, on the other hand, reduces the serological titre to about one-half and the infectivity to less than one-tenth of the original. For example, the preparation of tobacco I in Table I gave, after drying, a titre of 1/150,000 and at 10^{-5} and 10^{-6} gave an average of 41 and 5 lesions per leaf, and the preparation of the Rothamsted culture after drying gave a titre of 1/400,000 and no lesions at 10^{-5}. The preparations were dried unfrozen and the material dissolved readily in saline.

The serological relationships of these different cultures of tobacco necrosis viruses are summarized in Table II.

TABLE II.—*Serological Relationships of Different Cultures of Tobacco Necrosis Viruses.*

Culture.	Antiserum					
	Potato.	Tobacco I.	Tobacco II.	Rothamsted.	C.1.	C.2.
Potato	+	—	—	—	+	+
Tobacco I	—	+	+	—	—	—
Tobacco II	—	+	+	—	—	—
Tobacco VI	+	—	—	—	+	+
Princeton	+	—	—	—	+	+
Rothamsted	—	—	—	+	+	—

C. 1 is the serum used in the work of Pirie *et al.* (1938) and C. 2 the serum prepared against their crystalline product.

A + indicates that the particular culture has precipitated with that antiserum and a — that it has not, even though tests have been made over a wide range of antigen/antibody ratios in both constant antigen and constant antibody tests. It will be seen that the potato, tobacco VI and Princeton cultures are serologically related and all react with the same sera. Tobacco I and II are related to each other, but to no others, and are the only cultures not sero-

logically related to any of the constituents of the virus mixture investigated by Pirie *et al.* (1938). The Rothamsted culture is also serologically distinct. This reacts with the serum made against the bulk culture of Pirie *et al.* (1938), but not with the serum made against their crystalline material, confirming the suggestion made previously (Bawden, 1941) that this was a distinct virus from the amorphous material. It is likely that their crystalline product is very similar to, if not identical with, the culture here referred to as potato.

The rhombic dodecahedron is one of the more common forms in which proteins crystallize, e.g. bushy stunt virus, lysozyme and some of the haemoglobins and haemocyanins have all been found to form crystals of this type. Their occurrence in preparations of viruses causing tobacco necrosis, therefore, clearly cannot be taken as evidence of relationship between these and bushy stunt virus. However, to test this possibility, and to eliminate the chance of contamination, all preparations of tobacco cultures I and II which contained docecahedra were tested against antiserum to bushy stunt virus. None ever reacted. Similarly, tests with the other viruses causing tobacco necrosis have failed to show any serological relationships between these and bushy stunt virus.

SEDIMENTATION CONSTANTS.

The only evidence conflicting with the view that the crystalline virus isolated by Pirie *et al.* (1938) is the same as our potato culture is an apparent difference in the sedimentation constant. Dr. A. S. McFarlane, quoted by Pirie *et al.* (1938), found that this material was homogeneous with $S_w^{20^\circ} = 130 \times 10^{-13}$, whereas Dr. Ogston finds an $S_w^{20^\circ} = 116 \times 10^{-13}$ for our potato culture. Similar differences occur in the measurements made on bushy stunt virus (Bawden and Pirie, 1942) by Dr. McFarlane and Dr. Ogston in their centrifuges, and it is now agreed (*cf.* addendum to Bawden and Pirie, 1942) that the older values obtained by McFarlane were too high because of an uncertainty in the temperature of the rotor. Dr. Ogston also finds an $S_w^{20^\circ} = 116 \times 10^{-13}$ for our tobacco VI and Princeton cultures, which are serologically related to the potato culture. This is in reasonable agreement with the value of 112 given by Price and Wyckoff (1939) for the culture from which our Princeton one was derived.

The amorphous fraction of the preparations made by Pirie *et al.* (1938) was found by Dr. A. S. MacFarlane to have several components, the chief of which gave $S_w^{20^\circ} = 58$ and 220×10^{-13}. Similarly, the constants found by Dr. Ogston for our amorphous preparations of the Rothamsted culture differ from those for the other viruses. Except for one preparation, which contained a small amount of material with $S_w^{20^\circ} = 235 \times 10^{-13}$, these have been homogeneous and they have given $S_w^{20^\circ} = 49 \times 10^{-13}$. All preparations have had equal serological activity, and the preparation containing the heavy component was no more infective than the others. There is, therefore, no evidence that the heavy component is residual infective virus, but there is also no certainty that 49×10^{-13} is the sedimentation constant of fully active virus rather than of a non-infective derivative. With the other viruses that we have studied, loss of infectivity without loss of serological activity has not affected the sedimentation constant, but it cannot be assumed that this is also true of this

virus. If the value should turn out to be that associated with the active material, this will be the smallest plant virus on which centrifugal measurements have been made.

The yields of tobacco I and II cultures are so small that we have at no time had sufficient fresh material for centrifugation. Dr. Ogston's results on the other four cultures are set out in the Addendum.

CARBOHYDRATE AND PHOSPHORUS CONTENTS.

These viruses have not as yet been available in sufficient quantity for any detailed analytical work, but a fairly extensive series of carbohydrate (Pirie, 1936) and phosphorus (Kuttner and Lichtenstein, 1932) determinations have been made. No consistent differences between the different strains have been noticed and, with highly purified products, all values have fallen in the ranges —carbohydrate 7·0 to 8·5 per cent., phosphorus 1·7 to 2 per cent. The rates of colour development with the orcin reagent used in the carbohydrate estimation is the same with each strain, and the rate is compatible with the hypothesis that, as in the other plant viruses, the carbohydrate occurs in the form of a nucleic acid of the ribose type. The contaminants, other than inactive materials derived from the viruses, that are most frequently encountered in these preparations have a higher carbohydrate but a lower phosphorus content than the viruses. This gives these determinations some value as an index to the probable purity of a virus preparation.

SOLUBILITY.

Tobacco VI culture.—Purified preparations of this virus have never precipitated from solution in distilled water or dilute salt solution at any pH value between 3 and 8, even on prolonged standing.

When, on the other hand, crystals that have separated from ammonium sulphate solution are suspended in water or dialysed at pHs in the neighbourhood of 4·5 little of the virus goes into solution, and even after some days the fluid contains only 1 to 2 g. of virus per litre. The material which dissolves under these conditions has the same infectivity and serological activity as that which remains insoluble. At pH 8 a crystal suspension in saline dissolves immediately, and at pH 6·7 it dissolves in a few minutes. The rather critical pH range in which solution is quick is illustrated in Table III. The supernatant fluids were tested serologically and, under these conditions, a serum precipitation end-point of 1 : 128 represents substantially complete solution. At pH 5 the crystals dissolve on heating to 70° or on the addition of urea, but these treatments cause partial inactivation.

TABLE III.—*Solubility of Crystals of Tobacco VI at Different pHs.*

pH of crystal suspension.	Serological titre of the supernatant after 150 minutes at 0°.
6·4	1 : 128
6·1	1 : 64
5·7	1 : 32
5·3	1 : 16

The suspension contained 0·4 mg. of virus in the form of crystals in M/7 NaCl containing M/20 phosphate buffer at the pH stated.

Potato culture.—Solutions of this virus in distilled water or dilute salt solution do not precipitate at first at any pH between 3 and 8. Occasionally however, slightly acid solutions, in the absence of salt, have crystallized in the usual form after lying either at 0° or at room temperature for some months. This crystallization has always been incomplete and the crystals dissolve on the addition of a little salt, especially at about pH 8. There is no significant difference between the activities of the crystalline and soluble fractions separated in this way. The crystals that separate from ammonium sulphate solution will dissolve slowly after washing with distilled water or after dialysis. Solution seems to be most rapid at pH 4·5, although it occurs over a wide range, and it is favoured by cooling. It is noteworthy that a suspension of crystals at pH 4·5 will dissolve after a few days and then crystallize again after lying undisturbed for some months. We have no explanation for this phenomenon. The increase in clarity and stability in solutions of crystals when they are left at 0° for some hours has already been mentioned in the section on serology. It will be more fully investigated when more material is available.

Princeton culture.—We have studied the virus from this culture less fully than the others to which it is serologically related, but like them it has not precipitated from water or dilute salt solutions at any pH in the normal working range. We have failed to get indubitable crystals from this virus, but the precipitates produced by ammonium sulphate show a phenomenon comparable with the slow solution of the crystals from the tobacco VI and potato cultures. When the precipitates are taken up in water nothing can be sedimented by centrifuging at 4000 r.p.m., but the clarity of the suspensions increases progressively during the first few hours.

Tobacco cultures I and II.—Between pH 4 and 5 and in the absence of salts, precipitates invariably separate from solutions more concentrated than 0·2 per cent. and often separate from more dilute solutions. The precipitates redissolve readily on the addition of salt or on neutralization. Most of the activity of a preparation is found in the precipitate, suggesting that the virus itself has a low solubility, or is insoluble, at pH 4·5 in the absence of salts. However, several factors complicate this interpretation. Of the normal plant proteins still present most can be removed by dialysis at pH 6 followed by centrifugation at 0° C., and the precipitate chraracteristic of the preparations from these two cultures does not occur with the others, but the yields with tobacco I and II are so much smaller than with the others that the importance of contaminants is increased. The serological titres of freshly made preparations are sufficiently high to suggest that there is no gross contamination.

Rothamsted culture.—There is considerable uncertainty about the solubility of the virus from this culture. Our tests have all been made on preparations that have lost most of their infectivity, and no attempt has been made to ensure that the culture contains only one virus or virus strain. Also, the method of purification used is less rigorous. Precipitates usually separate on dialysis at pH 6–7, but these consist of normal leaf protein, have low phosphorus and carbohydrate contents and carry little or no activity. After

preparations have been freed from normal proteins by this treatment, they give a further precipitate when dialysed at about pH 4·5 ; as with preparations of tobacco I and II, the precipitate carries part of the activity with it. Precipitation is not complete, and most of the serological activity remains in the supernatant. The infectivity is usually fairly evenly divided, but occasionally we have had preparations in which almost all of the residual infectivity has appeared in the precipitate, although the serological activity has been predominantly in the supernatant. The precipitate dissolves on the addition of salt, or when brought to pH 7, and it has the same phosphorus and carbohydrate contents as the material that is soluble at pH 4·5.

BEHAVIOUR ON HEATING.

No significant difference has been found between the thermal inactivation point of the different cultures. In different tests the temperature at which ten minutes' heating has destroyed infectivity has varied between 75° C. and 85° C., the variations apparently depending more on the virus content of the preparation and the sensitivity of the test plants than on the virus culture. The results of one test using infective sap clarified by freezing and centrifuging are given in Table IV. It will be seen that heating at temperatures well below

TABLE IV.—*Effect of Heat on Tobacco Necrosis Viruses.*

Average number of lesions per leaf using infective sap diluted 1/1 and 1/50.

	Culture.							
	Potato.		Tobacco I.		Princeton.		Rothamsted.	
Temperature.	1/1.	1/50.	1/1.	1/50.	1/1.	1/50.	1/1.	1/50.
Unheated	147	125	113	96	155	152	190	157
50°	135	23	97	23	195	44	215	35
60°	24	3·5	26	2	29	3	19	1·5
70°	4	0	3	0	7	0·5	3·5	0
80°	1·5	0	0	0	1·5	0	0	0

the thermal inactivation point causes great loss of infectivity with all cultures. This loss is not accompanied by any corresponding fall in the serological titres, and by longer heating at these temperatures preparations can be rendered completely non-infective without any apparent change in their serological activity. None of these cultures has ever given any lesions after heating for ten minutes at or above 85° C., although the Princeton culture was derived from one for which Price (1938) found the thermal inactivation point to be 92° C. This is the temperature at which the viruses denature and lose their serological activity, and it is likely that with more concentrated inocula or more sensitive test plants some infectivity would be detected in preparations heated to this extent. Differences of this kind rather than differences in the heat stability of the viruses most probably account for these discrepancies (Table IV).

DISCUSSION.

The fact that similar symptoms are often caused by different viruses, while different symptoms are caused by related ones, has been one of the main reasons for the confusion that has arisen in the nomenclature of plant viruses.

It has also been an important factor in preventing the development of any system of classification, for it was early recognized (Johnson, 1926) that symptomatology alone provided no basis for this. Although there are no generally accepted criteria for classifying viruses, in recent years two tests have been widely used as methods for determining whether viruses causing different symptoms should be regarded as related strains. The first is the demonstration that a plant fully infected with one is protected against infection with the other, and the second is the demonstration that the two are serologically related. The first has been the more widely used, for serology as yet has had only limited applications, but wherever the two have been applied to the same viruses in critical tests, they have given the same results. In other words, wherever viruses have failed to protect plants against each other they have also failed to react with each other's antisera.

The plant protection method cannot be used as a test of relationships with these tobacco necrosis viruses, because they all produce identical symptoms and no host is known in which any becomes systemic. Therefore, serological tests remain the only standard method of indicating close relationships. These show that some of the cultures with which we have worked have antigens in common, whereas others are serologically distinct. Provided that conditions are such that a positive test is obtained with homologous sera, we regard a negative result with a heterologous serum as of greater significance than a positive result, for a positive test can only be taken as evidence of relationship if it is known that the antigen used to prepare the test serum was homogeneous. For example, if all our tests had been made using only the serum prepared by Pirie *et al.* (1938), the Rothamsted and potato cultures would appear to share antigens, for the immunizing antigen used to prepare this serum contained viruses from each culture. The antigen preparation used to prepare the serum to our potato culture was derived from a single local lesion, and we have no reason to believe that it contained more than one type of virus. Therefore the fact that tobacco VI and Princeton cultures react with it we take as evidence that they are serologically related, and we regard these and the potato culture as related strains of the same virus. Tobacco I and II and the Rothamsted culture, which do not react with this serum or with each other's sera, we regard as distinct viruses.

The grouping indicated by serological methods is supported by the other properties of the various cultures, for those that do not share antigens also differ more in other ways than those that do share antigens. The serologically related potato, tobacco VI, and Princeton cultures have been found to differ only in their behaviour when precipitated with ammonium sulphate, whereas, in addition to differences of this type, the serologically unrelated Rothamsted culture is less stable and tobacco I and II multiply less readily in infected plants. Tobacco I and II and the Rothamsted culture also differ from the others in their behaviour at pH 4·5 in the absence of salts. Further work may invalidate the relationships now indicated by serological methods, but at the moment it seems simplest, in view of the current usage of the terms, to regard tobacco necrosis as a disease that can be caused by distinct viruses, each of which may exist in a number of strains. In this respect, the disease resembles acronecrosis of the potato more than any other, but the different viruses and

virus strains that cause this disease can be distinguished by their reactions on a series of differential potato varieties, as well as by serological methods and plant protection tests (Bawden, 1936). In the same way it may be that the viruses and virus strains causing tobacco necrosis could be distinguished by studying their reactions on a wide range of hosts, for we have only worked with them on tobacco, *Nicotiana glutinosa*, and bean. What the relationships are between the various serologically unrelated viruses is as difficult to decide as with any other viruses that are serologically unrelated. It is obvious that all those with which we have worked do share some properties, but most of these are also shared by tomato bushy stunt virus, which also produces somewhat similar necrotic local lesions in bean and tobacco. We would therefore hesitate to suggest at this stage of our knowledge that the Rothamsted and tobacco I cultures are more closely related to each other, or to the potato culture, than they are to tomato bushy stunt virus, but it is obvious that all these do fall naturally into one group clearly distinct in most of their properties from other plant viruses that have been studied in any detail.

SUMMARY.

The purification of six separate cultures of viruses causing tobacco necrosis is described ; these cultures have been labelled Potato, Princeton, Tobacco VI, Rothamsted, Tobacco I and Tobacco II. The last two are probably identical, but the remainder differ in their properties, although they produce identical symptoms in tobacco and bean. The first three share antigens but are serologically unrelated to the others, suggesting that the disease can be caused by different viruses, each of which may occur in a number of strains. On precipitation with ammonium sulphate the products from four cultures have behaved systematically : Rothamsted gives an amorphous precipitate ; Princeton shows anisotropy of flow but gives no recognizable crystals ; potato crystallizes as thin lozenge-shaped plates ; and tobacco VI as hexagonal prisms. Tobacco I and II, for unknown reasons, crystallize in a variety of different forms. The two commonest are dodecahedra and bipyramids, but thin round laminae and elaborately twinned structures also occur. The materials isolated from all the cultures of tobacco necrosis seem to be essentially nucleoproteins.

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ADDENDUM: EXAMINATION IN THE ULTRACENTRIFUGE.

A. G. OGSTON.

(With Technical Assistance by W. WEINSTEIN.)

Seven preparations of tobacco necrosis viruses were examined in a Svedberg oil-turbine ultracentrifuge by the " diagonal schlieren " method (Philpot, 1938). The speed of rotation was 300 to 310 r.p.s.; 0·25 per cent. solutions of virus in 0·1 M NaCl and 0·01 M acetate buffer at pH 4·7 were contained in a 12 mm. cell. The results are given in Table I.

Table I.

Run.	Index number.	Sample.	$S_w^{20°} \times 10^{-13}$.
277	427Q	Potato culture	116
289	519D	Princeton culture	114
288	519F	,, ,,	116
286	514X	Tobacco VI culture	117
287	488U	Rothamsted culture	51·0
			240
290	488N	,, ,,	49·8
291	494T	,, ,,	49·4

All the samples appeared to be homogeneous, with the exception of 488U, a preparation of the Rothamsted culture, which contained a small proportion of a more rapidly sedimenting component. The sedimentation constant found here for the Princeton culture agrees with that given by Price and Wyckoff (1939) for the parent culture from which it was derived. The value found for the potato culture is different from that given by McFarlane, quoted by Pirie *et al.* (1938) for the crystalline fraction of their preparations, but it is likely that the earlier result was too high because of an uncertainty in the temperature of the centrifuge. A similar discrepancy has already been described in measurements made on preparations of bushy stunt virus carried out at about the same time (*cf.* addendum by Ogston to Bawden and Pirie, 1942).

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HIPPURIC ACID SYNTHESIS AS A TEST OF HEPATIC EFFICIENCY.

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A NUMBER of substances have been employed to determine the ability of the liver to detoxicate poisons; but sodium benzoate is the only one still in use. The test is widely used in the United States, but no series of cases has been reported here.

When sodium benzoate is ingested, or injected intravenously, the liver synthesizes glycine. This is conjugated with benzoic acid to form hippuric acid, which is excreted in the urine. Quick (1931) has shown that in the human subject hippuric acid is formed chiefly, if not entirely, in the liver. Quick (1932, 1933) was the first to employ hippuric acid synthesis after ingestion of sodium benzoate as a test of hepatic efficiency. The diminution in the amount of hippuric acid after damage to the liver is ascribed largely to diminished production of glycine, but possibly also to defective conjugation of glycine with benzoic acid.

The amount of hippuric acid excreted in the urine over a period of four hours after a dose of 6·0 g. of sodium benzoate (which is much more than enough to combine with all the glycine the healthy liver can synthesize in that time) measures the capacity of the liver to detoxicate sodium benzoate. The results of the test are usually expressed in terms of benzoic acid and not as hippuric acid. It is clear that the liver must be regarded as solely responsible for the synthesis of glycine, and also that care must be taken to prevent consumption of glycine-containing foods during the test, or immediately before it. In perhaps 10 per cent. of cases there are mild toxic effects—nausea, headache and vomiting. The last vitiates the test, and to meet this difficulty Quick, Ottenstein and Weltchek (1938) have devised a method of giving the sodium benzoate intravenously.

Apart from hepatic disease a low output of benzoic acid has also been reported in the following conditions: In congestive cardiac failure (Adlersberg and Minibeck, 1936; Yardumian and Rosenthal, 1937; Lindeboom, 1939) the formation of hippuric acid is prevented by disturbance of hepatic function. In chronic Bright's disease with azotaemia, formation of hippuric acid is normal, but it is retained in the blood stream like other nitrogenous waste products (Snapper and Greenbaum, 1924). A poor output of benzoic acid has

also been found in association with dehydration (White, Deutsch and Maddock, 1940). Lastly a low output in pernicious anaemia has been reported by Fouts, Helmer and Zerfas (1937), and by Barker (1938); but no direct relation with the level of haemoglobin has been determined.

In control subjects, patients who are not acutely ill and not suffering from cardiac failure or Bright's disease, the output of hippuric acid expressed as benzoic acid varies rather widely (Table II), ranging from 2·40 to 4·33 g. in four hours (Quick, 1933, 1936; Vacarro, 1935; Snell and Plunkett, 1936; Adlersberg and Minibeck, 1936; Lindeboom, 1939; Henderson and Splatt, 1942). All these workers adopted the shorter clinical method (Quick, 1936), which is much the most frequently employed and is used here.

Quick (1940) considered excretion of more than 3·6 g. of benzoic acid an abnormality, but could not explain it. Rosenberg and Soskin (1941) consider that " hyperexcretion " is evidence of damage to the liver so slight that it acts as a stimulus. Their evidence of such damage is not very convincing. Probstein and Lande (1941), using a slightly modified technique, obtained higher values in their controls than other workers, these controls consisting of healthy volunteers. Lastly Henderson and Splatt (1942) found in eight healthy persons a mean excretion of benzoic acid of 4·0 g. as against 3·3 g. in 17 hospital patients. From these findings it would appear that healthy, ambulant subjects excrete more benzoic acid than do convalescents, who have been almost exclusively used as controls. However, as the sufferers from liver disease are almost always in bed, it seems to the writer that patients who are also in bed convalescent from disease not involving the liver, heart, kidneys and haemoglobin level, do make a better control group than healthy ambulant individuals.

The commoner diseases of the liver fall into four groups: acute hepatitis, cirrhosis, obstruction of the common bile duct and secondary carcinoma. A survey of recent reports, in which hippuric acid synthesis has been used as a test of hepatic function, shows that in the great majority (Quick, 1933, 1936; Vacarro, 1935; Snell and Plunkett, 1936; Adlersberg and Minibeck, 1936; White, Deutsch and Maddock, 1939; Boyce, 1941; Bassett, Althausen and Coltrin, 1941; Henderson and Splatt, 1942) it was found defective in between 75 and 85 per cent. of cases. This compares well with other tests of hepatic efficiency. Up to 1939 McArdle (1940), in his review, found hippuric acid synthesis superior to any other. It is perhaps most frequently positive in secondary carcinoma of the liver, then in acute hepatitis, cirrhosis and obstructive jaundice, in that order. As far as disease of the biliary tract is concerned, cholecystitis, with or without stone, almost always gives normal results; but if cholangitis is present or stone in the common duct, the test is positive. More recent reports where several tests have been performed on the same patient are somewhat conflicting. Dye excretion tests, while undoubtedly very delicate, are of no value in the presence of jaundice. As far as the improved sugar tolerance tests are concerned, Bassett, Althausen and Coltrin (1941) have reported that the intravenous galactose tolerance test is less delicate. No comparison with the improved laevulose tolerance test has yet been reported. An empirical procedure, the cephalin cholesterol flocculation test (Hanger, 1939), has been reported as a more sensitive indicator of hepatic damage (Rosenberg and Soskin, 1941), but its specificity seems unproved.

Among the advantages claimed for the hippuric acid synthesis test are that it can be used in any form of hepatic disease with or without jaundice. It can be frequently repeated with a minimum of inconvenience to the patient and without entailing much work in the laboratory. Little real use of these attributes seems to have been made, however. By frequent repetition of the test Boyce (1941) observed improvement in surgical patients, after operation and after intensive treatment with glucose, but gives no protocols.

In diagnosis latent disease of the liver, such as cirrhosis, may occasionally be brought to light. In differential diagnosis of hepatic disease the test is of little help. Quick (1936) stated that diminished excretion of benzoic acid at an early stage in a case of jaundice indicated a toxic rather than an obstructive origin. Later workers have been unable to confirm this (White, Deutsch and Maddock, 1940 ; Boyce, 1941).

In prognosis an output of less than 1·5 g. of benzoic acid is taken by several workers to contra-indicate operation (Snell and Plunkett, 1936 ; White, Deutsch and Maddock, 1940). In general the amount of benzoic acid excreted is only roughly proportional to the degree of hepatic damage, and a low initial result does not necessarily imply permanent damage or a fatal issue. This is also illustrated in medical patients by the comparatively few observations in which the test has been repeated, generally only two or three times, on patients with acute hepatitis.

OBSERVATIONS.

Controls.

Observations have been made on 69 individuals who were free of hepatic, cardiac or renal disease and did not suffer from anaemia or any acute condition. For the most part they were convalescents, or suffered from a chronic disease of the nervous system. The shorter clinical method of Quick (1936) was used, the precipitate being weighed. A disadvantage of the method is that some of the hippuric acid remains in solution, stated by Quick (1933) to be 0·33 g. per 100 c.c. of urine. Allowance must be made for this. Quick (1933) stated that this method gave results in close agreement with the longer

TABLE I.—*Excretion of Benzoic Acid in Controls.*

Benzoic acid, g. in 4 hours.	Number of cases.
2·01–2·50	5
2·51–3·00	24
3·01–3·50	19
3·51–4·00	16
4·01–4·50	5
Total	69
Range	2·40–4·39 g.
Mean	3·19
Standard deviation	1·07 g.
,, error	±0·13 g.

procedure of extraction with ether. In this series of observations urine was collected at the end of four hours and not hourly (Boyce and McFetridge, 1938). By this means time was saved in the wards and in the laboratory and it was found that catheterization was very rarely necessary, as often happened when specimens were collected every hour for four hours. Table I shows the frequency-distribution of the results. The range was 2·40–4·39 g. of benzoic acid in four hours and the mean 3·19. The standard deviation was 1·07 and the standard error ± 0·13.

This is the largest series of controls yet published. It would appear that the range of normal excretion of benzoic acid is wider than that reported by previous workers ; but the highest and lowest figures correspond closely with those in Table II. It can also be seen that quite a number of controls excreted more than 3·6 g. of benzoic acid.

TABLE II.—*Excretion of Benzoic Acid in Controls.*

Author.	Benzoic acid, g. in 4 hours. Range.	Average.	No. of cases.
Quick, 1933, 1936 . . .	2·40–3·90	3·08	42
Vacarro, 1935	2·98–3·54	3·22	10
Snell and Plunkett, 1936 . .	2·55–3·30	—	11
Adlersberg and Minibeck, 1936 .	3·10–3·73	3·43	9
Lindeboom, 1939 . . .	2·97–4·33	3·70	30
Henderson and Splatt, 1942 .	2·60–4·10	3·30	17

Toxic effects.—Vomiting soon after the dose was not infrequent in this series, but unfortunately no record was kept. A fair proportion of those who vomited were able to retain the sodium benzoate at the second attempt next day. The intravenous administration of sodium benzoate was employed on a few occasions, but catheterization was required so frequently that the method was abandoned.

Repeated observations on nineteen controls.—A single normal result is accepted as evidence that serious liver damage is not present (Boyce and McFetridge, 1938 ; Quick, 1940). A single low observation is not accepted by the former authors as final, and the test is repeated. I have been unable to find any published figures to show what variations occur in repeated tests on the normal subject, and therefore on 19 of the controls 52 tests have been performed, on four occasions (3), on three (8), and on two (8).* In 15 controls the results lay within the normal range of 2·40–4·39 g. of benzoic acid in four hours. In four normal individuals a low output, 0·71, 1·32, 1·53 and 1·87 g. respectively, was obtained once in each subject. In all four, however, normal results were obtained either before or after the abnormal result. Each abnormal result was associated with a small volume of urine of less than 100 c.c., averaging 75 c.c. for the four abnormal tests as against 228 c.c. for the 48 normal results. These abnormal results are most probably due to failure to obtain all the urine passed over the period of the test, a defect to which all tests

* To economize space these results are not given in detail.

depending on collection of urine excreted over a fixed period are liable. Either the bladder is not emptied or some of the urine is lost. The point of practical importance is that tests giving low results should be repeated if the volume of urine passed in four hours is less than 120 c.c. This practice has been observed in this series of observations.

Hepatic Cases.

Acute epidemic hepatitis (Table III).—Sixty-four observations were made on 17 cases. Four patients, all of whom had a short and mild attack, as judged by the degree and duration of bilirubinaemia, showed normal excretion of benzoic acid (Cases 1–4). In all the others excretion of benzoic acid was

TABLE III.—*Excretion of Benzoic Acid in Acute Epidemic Hepatitis.*

Case No.	Date.	Plasma bilirubin, mg. per cent.	Benzoic acid, g. in 4 hours.	Duration in days.
1	1.xii.41	9·0	2·46	3
	8.xii.41	—	2·22	
	18.xii.41	1·5	1·73	
2	15.i.42	4·9	2·59	21
	21.i.42	2·0	2·47	
	29.i.42	0·6	4·19	
3	22.ii.42	5·8	2·58	11
	26.ii.42	1·0	3·01	
4	28.v.42	6·0	3·12	9
5	6.x.41	20·0	1·06	35
	10.x.41	—	0·81	
	20.x.41	—	0·79	
	28.x.41	3·0	2·20	
	1.xi.41	1·8	2·48	
	7.xi.41	—	2·03	
	24.xi.41	1·3	2·43	
6	3.x.41	16·2	0·85	10
	9.x.41	—	1·58	
	15.x.41	—	1·75	
	18.x.41	3·6	—	
	25.x.41	1·6	2·62	
7	8.iv.42	8·8	1·16	56
	16.iv.42	—	2·06	
	24.iv.42	3·2	2·82	
8	31.xii.40	8·4	1·31	28
	6.i.41	—	2·73	
9	6.iv.42	35·0	0·44	56
	10.iv.42	—	0·25	
	14.iv.42	26·0	0·65	
	22.iv.42	7·5	1·11	
	27.iv.42	—	0·60	
	2.v.42	3·0	0·74	
	7.v.42	3·0	0·74	
	14.v.42	1·4	0·92	
	15.v.42	—	1·46	
	22.vii.42	0·2	1·20	
10	1.vii.42	15·0	0·68	28
	9.vii.42	9·0	0·53	
	13.vii.42	2·7	0·83	
	21.vii.42	1·8	0·79	
	30.vii.42	1·8	1·39	
	4.viii.42	0·6	1·56	
	12.viii.42	0·6	0·92	
	20.viii.42	0·4	1·75	
	27.viii.42	0·5	1·84	
11	11.vii.42	20·0	0·16	12
	14.vii.42	20·0	0·28	
	23.vii.42	18.0	0.64	
	30.vii.42	18·0	0·84	
	4.viii.42	10·0	0·98	
	12.viii.42	3·0	0·92	
	20.viii.42	2·6	1·46	
	27.viii.42	1·8	1·72	
12	18.xi.41	22·0	0·89	8
	27.xi.41	—	1·86	
	2.xii.41	2·4	1·06	
	3.xii.41	—	1·68	
	4.xii.41	—	1·81	
13	17.vi.41	2·0	1·52	42
14*	27.v.41	6·2	1·34	5
	6.vi.41	—	1·51	
15	26.vi.41	2·0	2·05	? 60
16	29.v.41	6·2	1·15	28
	2.vi.41	—	1·34	
17	26.vi.41	12·0	0·60	240

* By chance this patient had been used as a control five months before, when he excreted 4·01 g. of benzoic acid.

reduced, slightly in one (Case 15), who was convalescent when first seen, greatly in the rest. In four patients (Cases 5–8) the amount of benzoic acid excreted returned fairly rapidly to normal, the time taken ranging from 5 to 11 weeks from the onset of the illness. In three patients (Cases 9–11), after prolonged periods of observation the amount of benzoic acid excreted was still considerably below the normal, although no excess of bile was present in the plasma and no abnormality of the liver could be detected on clinical examination. Acute hepatitis is accepted as one of the precursors of cirrhosis, and this persistent diminution in the detoxicating power of the liver may be an indication of permanent hepatic damage. To prove the point a much more prolonged study of such cases is clearly necessary. In four patients (Cases 12, 13, 14 and 16) the period of observation was too short to allow any conclusions to be drawn. Subacute necrosis seems probable in Case 17.

Acute toxic hepatitis (Table IV).—Excretion of benzoic acid was found to be diminished in hepatitis due to alcohol in excess, gold and arsenical injections and burns (Cases 18–22).

TABLE IV.—*Acute Toxic Hepatitis.*

Case No.	Date.	Plasma bilirubin, mg. per cent.	Benzoic acid, g. in 4 hours.	Remarks.
18	31.viii.41	4·8	0·47	7 weeks' duration. N.A.B.
19	6.v.42	—	0·65	3 weeks' duration. N.A.B.
20	6.ii.41	<0·2	1·98	Alcohol. Peripheral neuritis present.
21	25.iv.42	10·0	1·63	2 weeks' duration. Gold.
	4.v.42	5·5	1·41	—
	19.v.42	1·8	1·68	—
22	24.v.42	16·0	0·91	4 days' duration. Burns.
	28.v.42	—	3·35	—
	8.vi.42	0·9	3·63	—

TABLE V.—*Secondary Carcinoma of the Liver.*

Case No.	Date.	Plasma bilirubin, mg. per cent.	Benzoic acid, g. in 4 hours.	Case No.	Date.	Plasma bilirubin, mg. per cent.	Benzoic acid, g. in 4 hours.
23	12.ix.41	<0·2	0·31	30	25.ii.42	2·7	1·07
	9.x.41	<0·2	0·28	31	3.iii.42	<0·2	1·35
24	14.vii.41	0·2	1·50	32	26.ii.42	2·0	2·32
25	4.iv.41	9·6	1·66	33	19.vi.42	0·2	1·39
	24.iv.41	12·0	1·95	34	10.iii.42	<0·2	2·18
26	14.xii.41	1·0	1·50	35	8.viii.42	<0·2	1·99
27	25.v.41	<0·2	1·18	36	9.vi.42	0·2	1·49
28	6.ii.42	0·2	1·16		20.vi.42	<0·2	1·39
29	20.ii.42	1·4	1·41				

Secondary carcinoma of the liver (Table V).—In this condition it is the general experience that tests of hepatic efficiency seldom reveal loss of function. This is usually ascribed to the fact that much apparently normal tissue persists around the metastatic deposits. Most of those who have used the hippuric acid synthesis test, however, have obtained a very high percentage of positive

results. In this series of 14 cases excretion of benzoic acid was considerably reduced in twelve and just below normal in the remaining two. There was no relation between the amount of benzoic acid excreted and the plasma bilirubin.

TABLE VI.—*Cirhosis of the Liver.*

Case No.	Date.	Plasma bilirubin, mg. per cent.	Benzoic acid, g. in 4 hours.	Remarks.
37	20.x.41	1·3	1·73	No aetiological factor determined.
	7.i.42	2·2	0·84	
	12.ii.42	1·3	1·23	
38	20.vi.41	12·0	1·58	Ditto.
39	30.v.41	1·2	2·08	Jaundice 2 years before.
40	3.vii.41	0·2	0·94	Jaundice 3 years before.
	11.viii.41	3·0	1·21	
41	26.iii.42	0·6	1·92	Jaundice 9 months before.
42	11.ix.41	—	0·67	Jaundice 3 years before. Early primary carcinoma of liver as well as cirrhosis.
43	17.xi.42	1·2	2·89	Splenic anaemia.
44	2.ii.42	0·9	1·71	,, ,,
45	31.iii.42	0·4	1·09	,, ,,
46	7.ii.42	0·2	1·96	,, ,,
47	1.vi.42	2·5	1·52	Biliary cirrhosis of left lobe following traumatic stricture.
48	16.v.41	1·0	0·87	? Alcohol.
49	2.iv.42	1·8	0·56	,,
	6.iv.42	—	1·06	—
	16.iv.42	0·6	1·01	—
50	17.v.41	—	1·37	Haemochromatosis.

Cirrhosis of the liver (Table VI).—The series consists of 14 patients, in all of whom save one (Case 43) the excretion of benzoic acid was much reduced. The exception was a man with splenic anaemia. The spleen was large and the liver small. Ascites was present and he had had a sharp haematemesis. This exception cannot be explained. There was again no relationship between the plasma bilirubin and the excretion of benzoic acid. Four of the patients with cirrhosis had suffered from an illness labelled catarrhal jaundice nine months to three years before the onset of cirrhosis, and in them no other aetiological factor could be discovered.

Obstructive jaundice (Table VII).—Ten patients with obstructive jaundice due to carcinoma were investigated. Excretion of benzoic acid was greatly reduced in eight, slightly in one, and was normal in one. Surgical relief of the obstruction to the outflow of bile was followed by increased excretion of benzoic acid in Case 56 and, to a lesser degree, in Case 57. No change occurred in Case 52, where secondary invasion of the liver was found to have taken place. Large quantities of glucose daily failed to increase excretion of benzoic acid in Cases 55, 56 and 59. In this small series no relationship could be made out between the amount of benzoic acid excreted and the duration or the depth of the jaundice.

TABLE VII.—*Obstructive Jaundice.*

Case No.	Date.	Plasma bilirubin, mg. per cent.	Benzoic acid, g. in 4 hours.	Remarks.
51	25.vi.41	18·0	2·62	Carcinoma head of pancreas.
52	8.vii.41	17·0	2·05	,, ,, ,,
	13.xi.41	0·2	1·36	After cholecystenterostomy. Liver now grossly invaded.
53	28.xi.41	10·5	0·86	Carcinoma head of pancreas.
54	18.iv.41	15·0	1·56	,, ,, ,,
55	3.xi.41	16·0	0·33	,, ,, ,,
	17.xi.41	25·0	1·04	after much glucose by intravenous drip as well as by mouth.
	28.xi.41	21·0	1·03	
56	27.viii.41	16·0	0·33	Carcinoma head of pancreas.
	9.viii.41	—	1·01	After glucose therapy orally.
	21.viii.41	—	0·63	Ditto.
	10.x.41	1·6	1·56	3 days after cholecystjejunostomy.
	27.x.41	0·7	2·08	20 days after cholecystjejunostomy.
57	10.i.41	—	1·31	Carcinoma ampulla of Vater.
	15.i.41	—	1·20	—
	26.ii.41	—	1·82	10 days after cholecystgastrostomy.
58	25.v.42	12·5	0·92	Carcinoma head of pancreas
	3.vi.42	13·0	0·50	with metastasis to liver.
59	25.iii.42	—	1·15	Ditto.
	27.iii.42	11·5	0·96	—
	2.iv.42	17·0	1·13	Glucose by mouth without effect.
	6.iv.42	36·0	0·71	Ditto.
	13.iv.42	28·0	0·79	,,
60	12.viii.42	22·0	0·38	Carcinoma head of pancreas.

TABLE VIII.—*Cholecystitis ± Stone.*

Case No.	Date.	Plasma bilirubin, mg. per cent.	Benzoic acid, g. in 4 hours.	Case No.	Date.	Plasma bilirubin, mg. per cent.	Benzoic acid, g. in 4 hours.
61	13.v.42	6·0	0·59	70	1·iv·42	<0·2	2·52
	18.v.42	2·4	1·03	71	3.vi.42	<0·2	3·00
	31.v.42	2·4	0·83	72	5.vi.42	—	2·46
	20.vi.42	—	0·66	73	23.vi.42	2·1	2·42
62	31.vii.42	15·0	0·79	74	2.vii.42	—	2·58
	15.viii.42	3·0	0·37	75	12.vii.42	—	2·05
	24.viii.42	—	0·36	76	12.vii.42	—	2·32
63	11.vii.42	7·0	0·88	77	6.viii.42	—	3·42
	14.vii.42	3·0	1·18	78	9.viii.42	0·4	2·72
	19.vii.42	0·8	2·87	79	11.viii.42	—	2·49
64	23.vii.42	7·5	2·69	80	12.viii.42	0·3	3·97
65	1.v.42	—	2·49	81	21.viii.42	<0·2	2·87
66	29.xii.41	0·3	3·97	82	21.viii.42	<0·2	2·67
67	3.ix.41	0·2	2·96	83	2.ix.42	<0·2	2·50
68	7.v.41	—	2·48	84	5.ix.42	<0·2	2·90
69	1.iv.42	<0·2	2·04				

All cases except 81 and 82 showed gall-stones.

Case 61 = stones in common duct and gall-bladder, fever, jaundice and rigors for six months; much pus present at operation.

Case 62 = similar findings, fever, etc., for five weeks.

Cholecystitis ± stone (Table VIII).—Of the 24 patients in this group, two (Cases 61 and 62) showed gross and persistent deficiency in excretion of benzoic acid. Both patients had stones in the gall-bladder and in the common duct, and at operation much pus was present in the ducts. When a biopsy was made in Case 62 pus flowed from the cut in the edge of the liver. Glucose by mouth did not increase the amount of benzoic acid excreted by Case 61 ; but she did well, and was discharged from hospital without clinical manifestations of hepatic disease although benzoic acid excretion was unchanged. Case 62 died eight days after operation. The non-protein nitrogen of the blood was 126·0 mg. per cent. and she showed the picture of cholaemia. Two patients (Cases 63 and 64) also had stones in the gall-bladder and common duct ; but at operation no gross infection was present in the ducts. Case 63 showed a considerable but short-lived drop in excretion of benzoic acid. Of the remaining 20 patients only two showed a slightly diminished output. In disease of the biliary tract the site of the calculus and the presence or absence of cholangitis appear to determine the amount of benzoic acid excreted. Stones elsewhere than in the common duct and cholecystitis do not apparently diminish hepatic efficiency. All these patients underwent operation, and the liver appeared to be normal in all except Cases 61 and 62.

The results obtained with the five groups of diseases of the liver are summarized in Table IX.

TABLE IX.—*Results of Initial Tests on* 84 *Hepatic Cases.*

	Number of cases.	Excretion of benzoic acid g. in 4 hours.		
		<1·0.	1·0–2·4	>2·4.
Acute hepatitis	22	9	9	4
Metastatic carcinoma	14	1	13	0
Cirrhosis	14	4	9	1
Obstructive jaundice	10	5	4	1
Biliary tract disease	24	3	3	18
Totals	84	22	38	24

SUMMARY AND CONCLUSIONS.

1. The excretion of benzoic acid (as hippuric acid) in the urine after ingestion of sodium benzoate has been used as a test of hepatic efficiency in a wide range of hepatic diseases comprising acute hepatitis, cirrhosis, secondary carcinoma, obstructive jaundice and disease of the biliary tract.

2. In 69 " normal controls " the mean excretion of benzoic acid was 3·19 g. in four hours, the range being 2·40–4·39 g., with standard deviation of 1·07 and a standard error of 0·13 g.

3. On 84 patients with disease of the liver 164 observations have been made. In 54 of the 60 initial observations on patients with acute hepatitis, secondary carcinoma of the liver, cirrhosis and obstructive jaundice, the output of benzoic acid was less than 2·40 g. The cases were not specially selected and included mild or early lesions, so that the high proportion of positive

results shows clearly the delicacy of the test as a measure of hepatic efficiency. In disease of the biliary tract positive results were obtained when stone was present in the common duct, particularly if associated with cholangitis. In disease of the gall-bladder only, excretion of benzoic acid was normal or, in a small proportion of the cases, very slightly diminished. The appearance of the liver at operation was in agreement with the results of the test.

4. The test appears to be of value in the prognosis of acute hepatitis, since 3 out of 17 patients failed to excrete benzoic acid in normal amount after clinical recovery appeared to be complete. It is suggested that this may be an indication of the development of cirrhosis. Surgical relief of biliary obstruction resulted in an increased output of benzoic acid in two patients, but not in a third where metastatic invasion of the liver was present.

5. The test was of no value in differential diagnosis of hepatic disease or of jaundice.

6. Disadvantages of the test are (*a*) that in a small number of persons sodium benzoate excites vomiting; and (*b*) the difficulty of obtaining all urine excreted over a period of exactly four hours.

7. The test is however safe, easy to carry out, and it can be repeated frequently. It would appear to be the most sensitive test of hepatic efficiency available.

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THE ABSENCE OF A SEASONAL INFLUENCE UPON THE ROUS NO. 1 SARCOMA IN YOUNG CHICKS.

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THE fowl is unusual among laboratory animals in that many of its vital activities (ovulation, thyroid activity, moult, etc.) are controlled by seasonal influences. Consequently it may be suspected that a seasonal variation in cancer induction and growth would be exhibited in a more marked way than by the laboratory rodents usually employed for cancer research. There have been several reports that such an influence is evident.

Michalowsky (1928) stated that teratoma of the fowl's testis was readily induced by injection of zinc salts into the testis during spring, but not at other times of the year. The importance of the season in such experiments was also confirmed by Falin and Gromzewa (1939) and by Bagg (1936), who, however, was also able to induce teratomas at other seasons by simultaneous treatment with gonadotropic hormone. Peacock (1935) reported that the season had a marked influence upon the rate of growth and transmissibility of chemically-induced fowl sarcomas, the last six months of the year being the period of least activity of the tumours. The decrease in activity was so marked as to result in the loss of some strains of tumour. In a personal communication it was stated that this effect is still being shown. Peacock also noted that the loss of filterability of the Rous No. 1 sarcoma reported by Gye and Andrewes (1926) occurred during the season when chemically-induced tumours show least activity. Fränkel (1930) found transmission of Rous No. 1 by filtrates to be much easier during the egg-laying months of March, April, July and August. Recently Murphy and Sturm (1941) found that the interval between inoculation of a chemical carcinogen and the appearance of a tumour was greater during autumn than at other times of the year. On the other hand, many workers do not mention any seasonal variation, and ignore this factor in discussing their results. This suggests that in their experience it is not sufficiently important to influence their experiments.

In connection with investigations into the inheritance of the susceptibility of fowls to the Rous No. 1 agent, it became desirable to evaluate the possible effect of season upon the response of the 6-week-old chick to intramuscular inoculation with the free Rous agent. Records were available for the response of a large number of chicks of the Institute Brown Leghorn flock to inoculation with such material since November, 1935, and these were analysed to determine whether a seasonal variation was present. Only chicks aged 6–9 weeks

inoculated intramuscularly with a dose of agent of undoubted infectivity were considered. Many varied types of experiments were included, the use of cell-free Rous material being the only common factor. The early data are from the records of experiments performed by Dr. C. R. Amies, thus bringing the total number of birds considered to 971. The sexes were more or less equally represented in the experimental chickens.

The reactions to inoculation were grouped according to the following scheme: negative or regression; small tumour; large tumour. The size of the largest tumour 28 days after inoculation (or at death if caused by tumour before then) was taken to indicate the degree of susceptibility. Estimation of tumour size is to some extent subjective, but in the case of the Rous No. 1

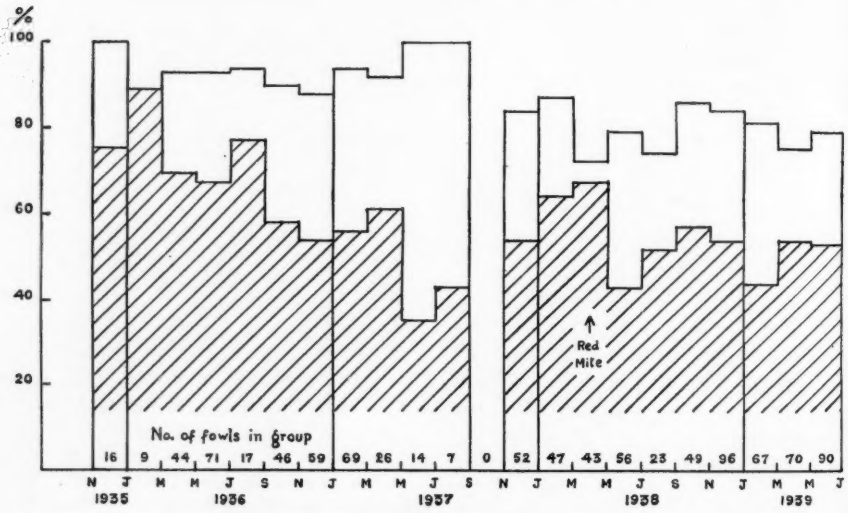

FIG. 1.—Showing the percentage of progressive tumours (white) and large tumours (shaded) induced each two-monthly period.

the two classes of size are readily separated in the vast majority of cases. The fowls were arranged in groups comprising birds inoculated during successive 2-monthly periods, which gave a reasonable number of animals in most groups.

A seasonal influence, if present, may affect either the establishment of a tumour, or its rate of growth. This would cause a variation in the number of progressive or large tumours respectively. The data are summarized in the figure. It will be seen that the numbers of progressive tumours remain fairly constant, and that the proportion of large tumours, though more variable, does not show a seasonal fluctuation.

Since 1941 work has continued upon the same stock of birds, and of the same age; only males were used in this period. The number used was fewer, and consequently the results are of lesser significance, and are not given in

detail. Again there was no indication of a seasonal influence. In this connection it should also be noted that the line of birds bred at this Institute for resistance to inoculation with the Rous agent (as determined from an inoculation given at 6 weeks of age) does not show a loss in resistance at any period of the year.

It is interesting to note that there is a drop in both curves in the spring and summer of 1938. This coincided with a heavy infestation by red mite, apparently acquired during transit to the Lister Institute (the only occasion when any large outbreak of disease or parasitism was encountered). As the infestation was prevented from spreading to all the birds, the effect upon the curves is only slight, but it was noted at the time that the tumour was much less active in the infested stock. This was to be anticipated, as it is known that ill-health of the host may reduce cancer growth (Rohdenburgh, 1918; Ewing, 1940). An example is provided by comparing the result of an experiment comprising 18 birds, kept in the infested animal house, with the average results for a number of birds determined about this time.

Tumour Response.

	Regression and negative.	+	++	+++	++++
Infested (per cent.)	22·2	50	11·1	5·5	11·1
Normal (per cent.)	12	15	17	31	25

DISCUSSION.

It is of interest that the fowls used in this work were from the same stock as many of those used by Peacock, in whose experiments a seasonal factor is marked. But the conditions are so different in the two types of work (use of agent-induced and non-filterable tumours, age of bird, etc.) that they are not in any way comparable. Indeed, it has been observed (Carr, 1942) that in adults of the resistant strain, the resistance to the Rous agent is modified by such seasonal factors as moult and laying. The present findings should therefore not be extended to conditions other than those which apply to this work. The absence of a seasonal variation in the conditions of this study is not surprising. After 30 years of passage the Rous tumour is probably near maximum virulence, and the immature chick has not yet developed the endocrine mechanism which responds to seasonal influences. In addition, the first six weeks of the chick's life are spent in an environment artificially maintained at optimum conditions. A significant seasonal variation in such chicks does exist (Galpin, 1939), but is of less importance than individual variations.

The decrease in tumour growth associated with parasitism is of some interest. It is obvious that in a parasitized stock the degree of parasitism will vary according to seasonal changes or alterations in experimental conditions, and thus induce a variation in the tumour growth in the hosts. The importance of using disease-free stocks in such studies cannot be stressed too highly.

SUMMARY.

No definite seasonal variation in the susceptibility to the Rous No. 1 sarcoma has been found in healthy young Brown Leghorn chickens. It is noted that parasitic infestation may produce a seasonal alteration in susceptibility.

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SURVEY OF PAPERS.

A. PIRIE has investigated the hyaluronidase content of tumours. A weak enzyme has been found in some mammalian and one fowl tumour. The Rous and Fujinami fowl tumours contain no hyaluronidase but contain a viscous polysaccharide which is hydrolyzed by this enzyme. Digestion of this polysaccharide by hyaluronidase does not increase the yield of virus extractable from the tissue. Preparations of this viscous polysaccharide may contain copper (p. 277).

MITCHELL describes methods whereby the effect of X- and gamma radiations on the nucleic acid metabolism of tissues can be measured by ultraviolet photomicrography. From measurements of the amount of blackening in photographs of sections of tissues before and after radiation, changes in the concentration of nucleic acid can be deduced. Additional information is provided by measurements of the ultraviolet absorption and fluorescence spectra and by histochemical tests (p. 285).

By these methods he has shown that X- and γ-radiation produce in proliferating and differentiating cells a disturbance of nucleic acid metabolism of which one aspect is the accumulation of pentose nucleotides in the cytoplasm of the affected cells. These nucleotides are similar to those found normally, but in much lower concentration, in many rapidly growing cells, and can be assumed to accumulate as a result of a disturbance of the normal metabolic processes either by increased rate of formation or decreased rate of removal (p. 296).

At the same time there is no comparable increase in the nucleic acid content of the nuclei after irradiation. A probable explanation is that irradiation produces changes within the nucleus or in the nuclear membrane, and thus inhibits the transference of ribo-nucleotides from the cytoplasm into the nucleus, where the final stages of the normal synthesis of thymonucleic acid takes place. These results appear to offer a simple explanation of the histological changes observed after X- and γ-radiation. Inhibition of the synthesis of thymonucleic acid provides a mechanism for the well-known inhibition of mitosis. The accumulation of ribo-nucleotides in the cytoplasm may account for the main cytoplasmic changes (p. 309).

BAWDEN AND N. W. PIRIE have purified six separate cultures of viruses causing tobacco necrosis. Two are probably identical, but the others differ in their properties, although they produce identical symptoms in tobacco and bean. Three of the viruses share antigens, but are serologically unrelated to the others, suggesting that the disease can be caused by different viruses, each of which may occur in a number of strains (p. 314).

RENNIE has investigated the hippuric acid synthesis test and finds that it is the most sensitive test of hepatic efficiency available (p. 329).

CARR has found that there is no definite seasonal variation in the susceptibility to the Rous No. 1 sarcoma in healthy young Brown Leghorn chickens. Parasitic infestation of the chickens, however, may produce a seasonal reduction in susceptibility (p. 339).